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Établissement de fonctions de corrections relatives aux masses volumiques de divers liquides œnologiques

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Résumé

Il existe des tables de corrections permettant d'exprimer les mesures densimétriques relatives aux moûts et aux vins en d_{20}^{20}. Elles sont officialisées depuis 1971 par la Communauté Économique Européenne. Ces tables nécessitent souvent une double interpolation (densité-température ou titre alcoométrique-température).

Notre travail porte sur la possibilité de remplacer les diverses tables de corrections par des fonctions simples évitant à l'utilisateur toute interpolation.

Les résultats obtenus sont en bon accord avec ceux donnés par les tables.

Mots clés : *masse volumique, matrice, norme cuclidienne.*

I. — Introduction

Pour normaliser les mesures densimétriques, la Communauté Économique Européenne officialisait en 1971 la température de 20 °C. Des tables de corrections ont été établies pour exprimer toutes les mesures en d_{20}^{t} à partir d'une correction nulle pour la d_{20}^{20}.

Si l'utilisation de ces tables ne pose pas de problèmes majeurs au laboratoire, il n'en est pas de même pour les opérations de contrôle.

Aussi nous a-t-il semblé intéressant de mettre sous forme de fonctions simples, les différentes corrections à apporter aux mesures densimétriques. Le terme correctif obtenu est fonction de deux paramètres : température et masse volumique ou titre alcoométrique.

II. — Étude des tables de correction

1. — *Méthode graphique*

A partir des tables de corrections, publiées au Journal Officiel des Communautés Européennes, établies avec un pycnomètre en pyrex, nous avons tracé pour une température donnée, les variations de la correction en fonction soit de la masse volumique (moûts naturels ou concentrés, vins de liqueur, mélanges hydroalcooliques) soit du titre alcoométrique centésimal (vins secs).

La linéarisation de ces fonctions est un moyen commode pour rendre compte de la correction à apporter aux masses volumiques. L'expression générale de cette fonction est de la forme :

$$C = A_1 \ (T - 20) \ (X - A_2)$$

C représente la correction à apporter à une température T et pour une masse volumique ou titre alcoométrique centésimal X, A_1 et A_2 sont des cœfficients constants.

Selon que les températures sont supérieures ou inférieures à 20 °C, les courbes présentent des pentes positives ou négatives : on aura donc deux fonctions de corrections par table.

Les figures (1), (2), (3) et (4) traitent des différents cas étudiés. Pour ne pas augmenter le nombre de graphes, nous ne donnons ici qu'un seul exemple traitant le cas des vins de liqueur (13° alcool).

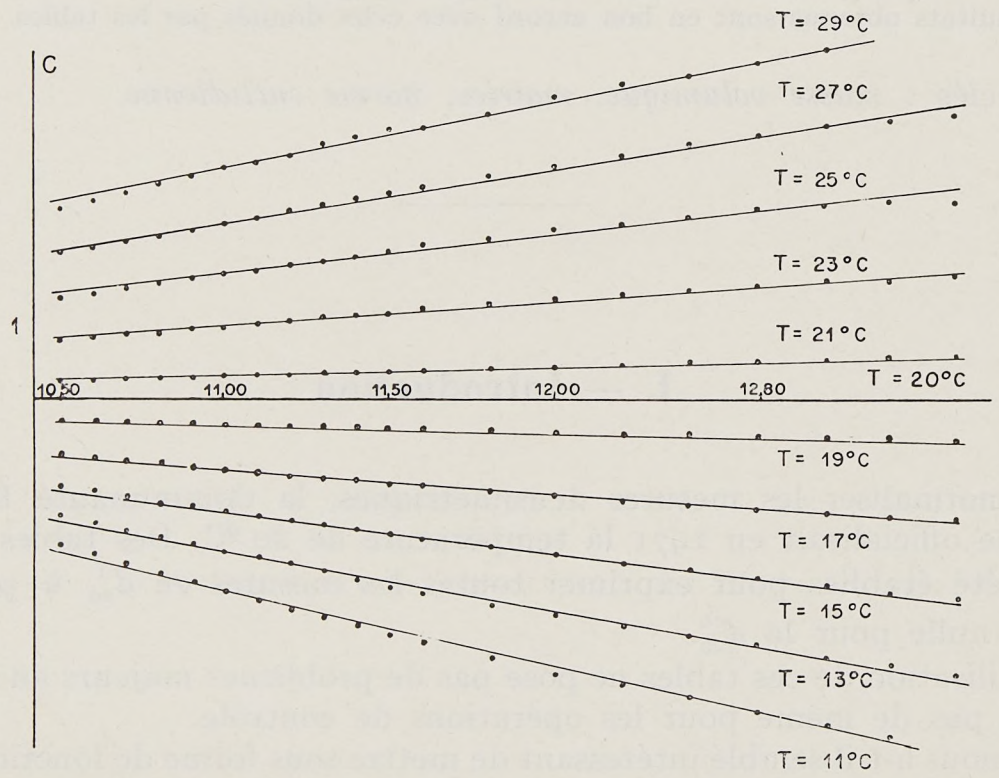


FIG. 1. — *Cas des moûts naturels et concentrés*
Droite représentant les variations de la correction de la masse volumique pour différentes températures.

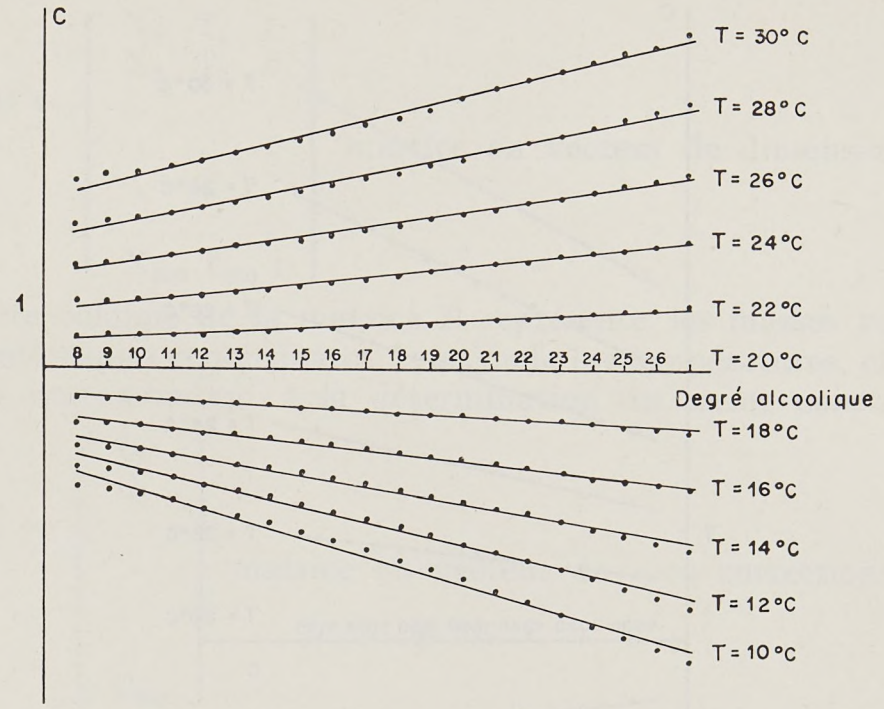


FIG. 2. — *Cas des vins secs*
Droites représentant les variations de la correction de la masse volumique pour différentes températures à un degré alcoolique donné.

2. — *Méthode mathématique*

Les paramètres C, X, T, déterminent un espace à trois dimensions. A chaque température, pour un demi-tableau donné, correspond une courbe assimilée à une droite $C_T = f(X_T)$ inscrite dans un plan fixé par T. Ces droites ayant pratiquement même pente, le but de la méthode mathématique utilisée est de trouver un plan moyen, englobant toutes ces droites, et d'équation générale :

$$C = A_1(T - 20)\ (X - A_2)$$

ou bien encore :

$$C = a_1.T + a_2.X + a_3$$

Afin de ne pas dépasser la capacité de calcul de l'ordinateur, nous nous sommes limités à deux cent données.

Soit C_j la correction correspondant à la masse volumique ou au titre alcoométrique centésimal X_j, à la température T_j. Nous recherchons à obtenir les cœfficients constants a_1, a_2, a_3 de telle façon que :

$$C_j = a_1T_j + a_2X_j + a_3$$

avec j variant de 1 à 200.

Nous avons ainsi 200 équations à 3 inconnues. Ce système surabondant d'équations linéaires à cœfficients réels sera résolu par la méthode des moindres carrés.

On pose :

$$A = \begin{bmatrix} a_1 \\ a_2 \\ a_3 \end{bmatrix}$$ matrice ou vecteur à 3 composantes

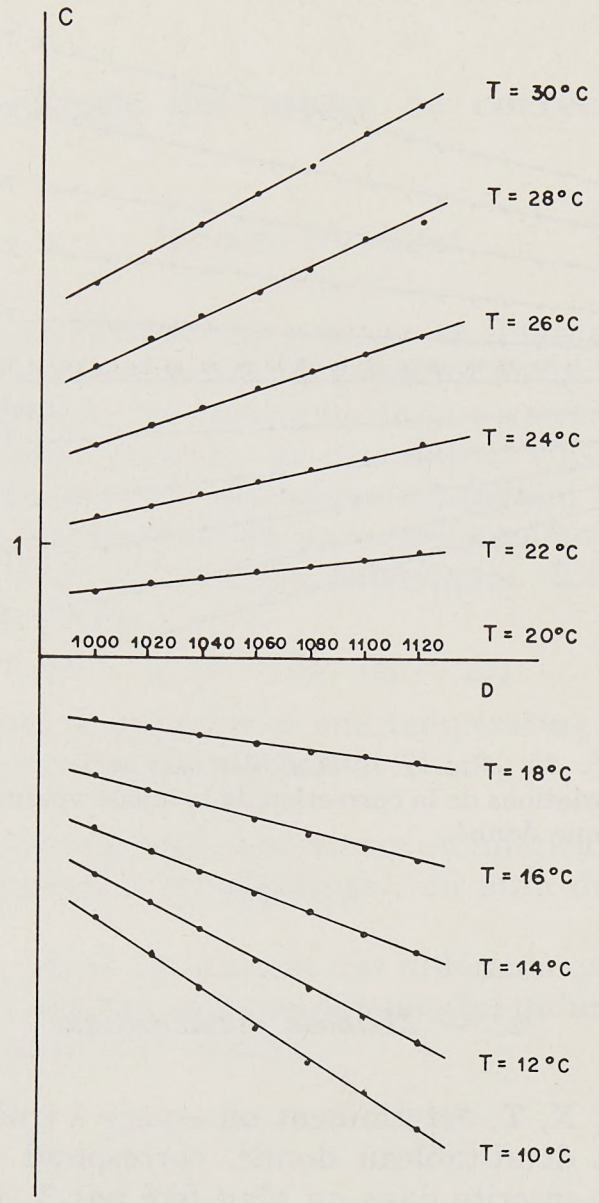


FIG. 3. — *Cas des vins de liqueur à 13°*
Droites représentant les variations de la correction de la masse volumique pour différentes températures.

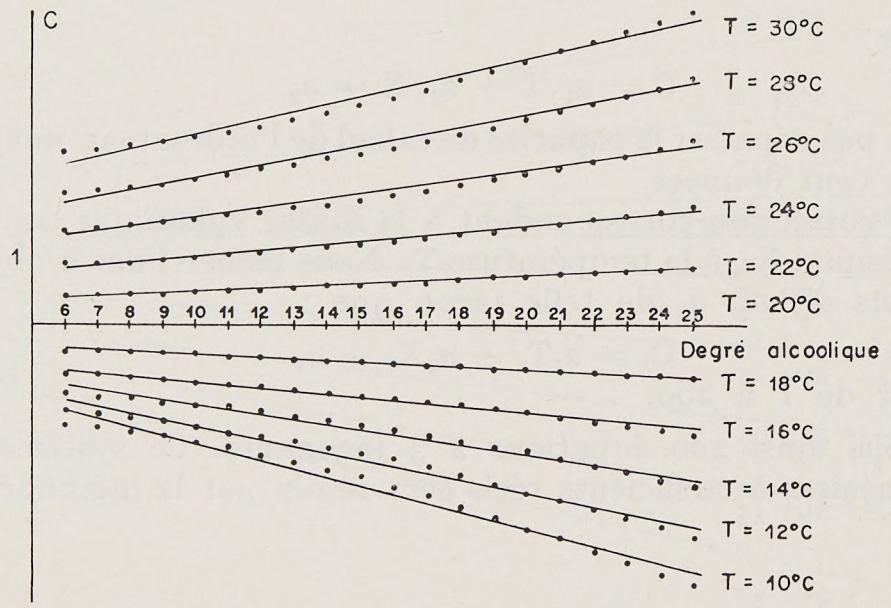


FIG. 4. — *Cas des mélanges hydroalcooliques*
Droites représentant les variations de la correction de la masse volumique pour différentes températures à un degré alcoolique donné.

$$B = \begin{bmatrix} X_1 & T_1 & 1 \\ X_2 & T_2 & 1 \\ . & . & . \\ . & . & . \\ . & . & . \\ . & . & . \\ X_{200} & T_{200} & 1 \end{bmatrix}$$ matrice ou vecteur de dimension 200×3

La première colonne de la matrice B représente les masses volumiques ou les titres alcoométriques centésimaux, la seconde les températures, et la troisième le cœfficient 1 correspondant à la détermination du terme constant.

$$C = \begin{bmatrix} C_1 \\ C_2 \\ . \\ . \\ . \\ . \\ C_{200} \end{bmatrix}$$ matrice ou vecteur des 200 corrections

La méthode des moindres carrés revient à déterminer le vecteur A tel que la distance de tous les points au plan moyen soit minimale, c'est-à-dire que la norme euclidienne $\| BA - C \|$ soit minimale :

$$d = \| B.A - C \| = \left[\sum_{j=1}^{200} (a_1 X_j + a_2 T_j + a_3 - C_j)^2 \right]^{1/2}$$

$$\Leftrightarrow d^2 = \| BA - C \|^2 = \left[\sum_{j=1}^{200} (a_1 X_j + a_2 T_j + a_3 - C_j)^2 \right]$$

Pour que d^2 soit minimum, il suffit que :

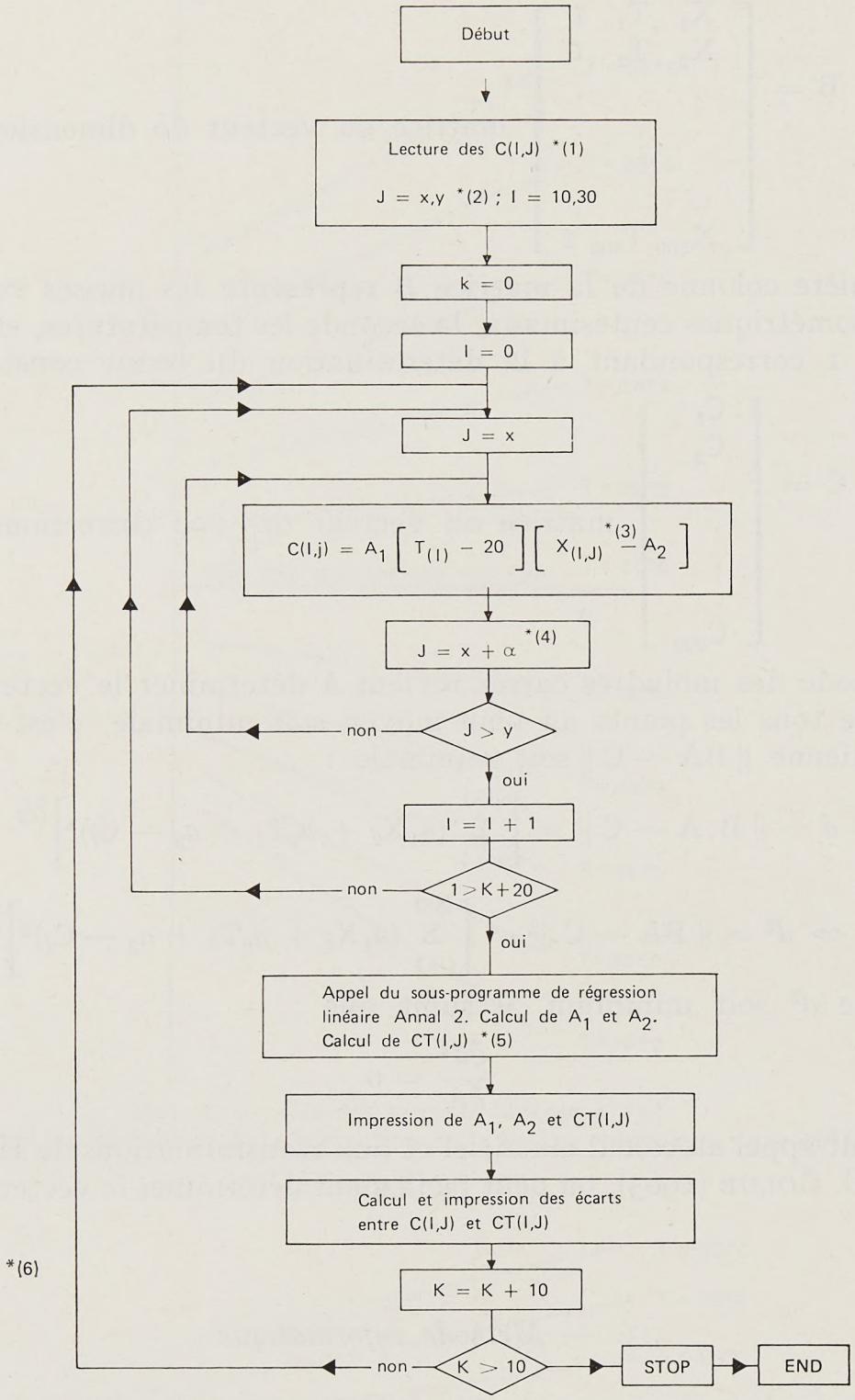
$$\frac{\partial d^2}{\partial A} = 0$$

En faisant appel au calcul matriciel et aux transformations de HOUSEHOLDER décrites par G. GOLUB (1965), on peut facilement déterminer le vecteur A par ordinateur.

3. — *Méthode informatique*

Pour obtenir la précision souhaitée tout en respectant la capacité de calcul de l'ordinateur (7 600 Control Data) nous avons limité les plages de températures (10 à 20 °C) ainsi que celles des masses volumiques ou des titres alcoométriques centésimaux.

L'organigramme porte sur les moûts naturels et concentrés, les vins secs, les vins de liqueur et les mélanges hydroalcooliques :



*(1) : C(I, J) représente les corrections données par la table.
*(2) : (J = x, y J varie de x à y,
(I = 10,30 I varie de 10 °C à 30 °C.
N.B. : *pour les moûts :* x = masse volumique de 1,05,
y = masse volumique de 1,32;
pour les vins secs : x = 8° d'alcool,
y = 27° d'alcool;
pour les vins de liqueur : x = *masse volumique* 1,
y = masse volumique 1,12.
*(3) : X(I, J) représente :

— Une masse volumique pour les moûts, les vins de liqueur et les mélanges hydroalcooliques,
— Un titre alcoométrique centésimal pour les vins secs.

*(4) : α représente le pas de progression de l'indice J =

— α = 1° d'alcool pour les vins secs;
— α = 0,01 puis 0,02 de masse volumique pour les moûts;
— α = 0,02 de masse volumique pour les vins de liqueur.

*(5) : CT(I, J) représente les corrections calculées
*(6) : Cette boucle permet de passer des températures < 20 °C aux températures > 20 °C.

III. — Résultats

1. — *Les différentes fonctions de correction obtenues*

a. *Moûts naturels et concentrés*

$$T < 20\ ^0C : C = 1{,}265\ (\rho - 0{,}845)\ (T - 20)$$
$$T > 20\ ^0C : C = 1{,}112\ (\rho - 0{,}755)\ (T - 20)$$

b. *Les vins secs*

$$T < 20\ ^0C : C = 0{,}014\ (^0A + 4{,}018)\ (T - 20)$$
$$T > 20\ ^0C : C = 0{,}012\ (^0A + 12{,}617)\ (T - 20)$$

c. *Les vins de liqueur*

13 °A	$T < 20\ ^0C : C = 1{,}596\ (\rho - 0{,}842)\ (T - 20)$ $T > 20\ ^0C : C = 1{,}383\ (\rho - 0{,}758)\ (T - 20)$
15 °A	$T < 20\ ^0C : C = 1{,}596\ (\rho - 0{,}825)\ (T - 20)$ $T > 20\ ^0C : C = 1{,}383\ (\rho - 0{,}741)\ (T - 20)$
17 °A	$T < 20\ ^0C : C = 1{,}595\ (\rho - 0{,}807)\ (T - 20)$ $T > 20\ ^0C : C = 1{,}375\ (\rho - 0{,}719)\ (T - 20)$
19 °A	$T < 20\ ^0C : C = 1{,}586\ (\rho - 0{,}785)\ (T - 20)$ $T > 20\ ^0C : C = 1{,}372\ (\rho - 0{,}697)\ (T - 20)$
21 °A	$T < 20\ ^0C : C = 1{,}589\ (\rho - 0{,}764)\ (T - 20)$ $T > 20\ ^0C : C = 1{,}383\ (\rho - 0{,}678)\ (T - 20)$

d. *Mélanges hydroalcooliques*

$$T > 20\ ^0C : C = -\ 0{,}01227\ (\rho - 1\ 001{,}765)\ (T - 20)$$
$$T < 20\ ^0C : C = -\ 0{,}00935\ (\rho - 1\ 011{,}834)\ (T - 20)$$

Dans toutes ces relations, ρ indique la masse volumique à la température T donnée par les tables.

2. — *Validité des fonctions de correction*

Toutes ces fonctions de correction ont été testées à l'ordinateur. Les tables (1), (2), (3), (4) et (5) indiquent les écarts pour certaines températures entre la correction donnée par la table et celle calculée par la fonction.

TABLEAU I

Moûts concentrés et naturels

30 °C	25 °C	21 °C	19 °C	15 °C	10 °C	T
Ecarts	Ecarts	Ecarts	Ecarts	Ecarts	Ecarts	Masses volumiques
— 0,08	— 0,13	— 0,04	— 0,03	+ 0,02	0,28	1,05
— 0,04	— 0,10	— 0,04	— 0,04	— 0,00	0,24	1,06
— 0,01	— 0,09	— 0,03	— 0,04	— 0,02	0,18	1,07
0,03	— 0,07	— 0,02	— 0,04	— 0,03	0,15	1,08
0,05	— 0,05	— 0,02	— 0,05	— 0,05	0,11	1,09
0,08	— 0,04	— 0,01	— 0,05	— 0,06	0,09	1,10
0,10	— 0,02	— 0,01	— 0,05	— 0,07	0,05	1,11
0,11	— 0,01	— 0,01	0,05	— 0,08	0,04	1,12
0,13	0,01	— 0,01	— 0,06	— 0,09	0,01	1,13
0,15	0,02	— 0,01	— 0,06	— 0,10	0,00	1,14
0,16	0,03	0,00	— 0,05	— 0,11	0,02	1,15
0,17	0,05	0,01	— 0,05	— 0,12	— 0,03	1,16
0,18	0,06	0,01	— 0,06	— 0,12	— 0,04	1,18
0,17	0,06	0,01	— 0,05	— 0,12	— 0,03	1,20
0,22	0,05	0,01	— 0,05	— 0,11	— 0,02	1,22
0,12	0,02	0,02	— 0,04	— 0,09	— 0,01	1,24
0,07	0,01	0,02	— 0,04	— 0,07	0,07	1,26
0,00	— 0,03	0,01	— 0,02	— 0,04	0,08	1,28
— 0,10	— 0,08	— 0,01	— 0,01	— 0,00	0,19	1,30
— 0,20	— 0,15	— 0,02	+ 0,00	— 0,03	0,28	1,32

On constate que ces écarts portent en général sur la cinquième décimale, la quatrième n'étant atteinte que pour les températures les plus éloignées de 20 °C. *N.B.* : L'écart calculé pris avec son signe doit être ajouté à la correction calculée pour obtenir la correction donnée par la table qui affecte toujours la troisième décimale. Lorsque le nombre représentant l'écart calculé n'a pas de signe, celui-ci sous-entend un signe plus.

TABLEAU 2

Vins secs et vins débarassés d'alcool

30 °C	25 °C	21 °C	19 °C	15 °C	10 °C	T / degré alcool
Ecarts	Ecarts	Ecarts	Ecarts	Ecarts	Ecarts	
+ 0,29	0,00	— 0,03	— 0 06	— 0 18	— 0 09	8
+ 0 22	— 0 03	— 0 03	— 0 05	— 0 14	— 0 02	9
+ 0 18	— 0 05	— 0 03	— 0,04	— 0,11	+ 0,05	10
+ 0,13	— 0,07	— 0,03	— 0,04	— 0,08	+ 0,09	11
+ 0,10	— 0,09	— 0,04	— 0,04	— 0,07	+ 0,13	12
+ 0,07	— 0,10	— 0,04	— 0,03	— 0,05	+ 0,16	13
+ 0,05	— 0,11	— 0,04	— 0,03	— 0,04	+ 0,18	14
+ 0,03	— 0,11	— 0,04	— 0,02	— 0,03	+ 0,20	15
+ 0,02	— 0,12	— 0,05	— 0,02	— 0,02	+ 0,20	16
+ 0,02	— 0,12	— 0,05	— 0,03	— 0,01	+ 0,21	17
+ 0,02	— 0,11	— 0,04	— 0,02	— 0,01	+ 0,20	18
+ 0,02	— 0,11	— 0,03	— 0,02	— 0,02	+ 0,19	19
+ 0,04	— 0,11	— 0,04	— 0,02	— 0,02	+ 0,17	20
+ 0,06	— 0,10	— 0,04	— 0,02	— 0,33	+ 0,15	21
+ 0,08	— 0,08	— 0,03	— 0,03	— 0,04	+ 0,12	22
+ 0,11	— 0,06	— 0,03	— 0,03	— 0,06	+ 0,08	23
+ 0,13	— 0,04	— 0,03	— 0,03	— 0,07	+ 0,05	24
+ 0,16	— 0,03	— 0,03	— 0,03	— 0,09	+ 0,00	25
+ 0,10	— 0,01	— 0,02	— 0,04	— 0,11	— 0,05	26
+ 0,23	+ 0,01	— 0,01	— 0,04	— 0,13	— 0,10	27

Ex : Cas des moûts

1°) $\rho = 1{,}07$ à 10 °C

$C = 1{,}265(1{,}07 - 0{,}845)\ (10 - 20)$

$C = 2{,}84\ 10^{-3}$

L'écart trouvé est de 0,18,

soit : $(-2{,}84 + 0{,}18)\ 10^{-3} = -2{,}66\ 10^{-3}$

qui correspond exactement à la correction donnée par la table.

TABLEAU 3

Vins de liqueur

Vins de 13°

30 °C	25 °C	21 °C	19 °C	15 °C	10 °C	T / Masses volumiques
Ecarts	Ecarts	Ecarts	Ecarts	Ecarts	Ecarts	
0,10	— 0,06	— 0,03	— 0,05	— 0,06	0,15	1,000
0,08	— 0,06	— 0'03	— 0,06	— 0,07	0,12	1,020
0,09	— 0,05	— 0,03	— 0,05	— 0,08	0,09	1,040
0,11	— 0,04	— 0,02	— 0,05	— 0,08	0,05	1,060
0,09	— 0,04	— 0,02	— 0,05	— 0,07	0,07	1,080
0,07	— 0,03	— 0,01	— 0,05	— 0,07	0,15	1,100
0,06	— 0,03	— 0,01	— 0,05	— 0,05	0,11	1,120

Vins de 15°

0,09	— 0,06	— 0,03	— 0,05	— 0,06	0,15	1,000
0,08	— 0,06	— 0,03	— 0,06	— 0,07	0,12	1,020
0,09	— 0,05	— 0,02	— 0,06	— 0,07	0,07	1,040
0,10	— 0,04	— 0,01	— 0,06	— 0,08	0,07	1,060
0,10	— 0,04	— 0,01	— 0,05	— 0,07	0,08	1,080
0,08	— 0,03	— 0,01	— 0,05	— 0,06	0,09	1,100
0,05	— 0,03	— 0,01	— 0,05	— 0,04	0,12	1,120

2°) $\rho = 1{,}07$ à 25 °C

$C = 1{,}112(1{,}07 - 0{,}755)\ (25 - 20)$

$C = 1{,}75\ 10^{-3}$

L'écart trouvé est — 0,09,

soit : $(1{,}75 - 0{,}09)\ 10^{-3} = 1{,}66\ 10^{-3}$

qui correspond exactement à la correction donnée par la table.

TABLEAU 4

Vins de liqueur Vins de 17°

30 °C	25 °C	21 °C	19 °C	15 °C	10 °C	T / Masses Volumiques
Ecarts	Ecarts	Ecarts	Ecarts	Ecarts	Ecarts	
0,08	— 0,06	— 0,04	— 0,05	— 0,06	0,15	1,000
0,08	— 0,06	— 0,02	— 0,05	— 0,07	0,11	1,029
0,09	— 0,05	— 0,02	— 0,05	— 0,07	0,08	1,040
0,10	— 0,04	— 0,02	— 0,06	— 0,07	0,06	1,060
0,10	— 0,03	— 0,02	— 0,05	— 0,06	0,07	1,080
0,08	— 0,03	— 0,01	— 0,05	— 0,05	0,08	1,100
0,05	— 0,03	— 0,01	— 0,04	— 0,03	0,11	1,120
			Vins de 19°			
0,08	— 0,06	— 0,04	— 0,05	— 0,05	0,14	1,100
0,08	— 0,06	— 0,02	— 0,05	— 0,06	0,11	1,020
0,09	— 0,04	— 0,02	— 0,05	— 0,07	0,08	1,040
0,10	— 0,03	— 0,02	— 0,05	— 0,07	0,06	1,060
0,10	— 0,03	— 0,02	— 0,05	— 0,06	0,06	1,080
0,08	— 0,03	— 0,01	— 0,05	— 0,05	0,08	1,100
0,05	— 0,02	— 0,01	— 0,04	— 0,03	0,11	1,120

TABLEAU 5

Vins de liqueur Vins de 21°

30 °C	25 °C	21 °C	19 °C	15 °C	10 °C	T / Masses volumiques
Ecarts	Ecarts	Ecarts	Ecarts	Ecarts	Ecarts	
+ 0,08	— 0,05	— 0,04	— 0,04	— 0,05	0,14	1,000
+ 0,08	— 0,05	— 0,02	— 0,05	— 0,06	0,10	1,020
+ 0,10	— 0,04	— 0,02	— 0,05	— 0,06	0,07	1,040
+ 0,10	— 0,03	— 0,02	— 0,05	— 0,07	0,05	1,060
+ 0,09	— 0,02	— 0,02	— 0,05	— 0,06	0,06	1,080
+ 0,07	— 0,02	— 0,01	— 0,05	— 0,05	0,08	1,100
+ 0,04	— 0,02	— 0,01	— 0,04	— 0,03	0,10	1,120

Conclusion

La méthode de calcul suivie ainsi que l'établissement d'un programme informatique approprié nous ont permis de formuler des équations donnant directement la correction à apporter aux mesures des masses volumiques pour une température donnée.

Les résultats obtenus sont en bon accord avec la précision nécessaire à la mesure.

La programmation de ces fonctions et leur traitement par un microprocesseur de poche, évitent à l'utilisateur les interpolations inhérentes aux tables de corrections et de manipulations peu pratiques.

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Summary

Musts and wines: corrected specific gravity

Corrections tables allow us to give specific gravity d_{20}^{20} for musts and wines. This tables are made public from 1971 for C.E.E. A double interpolation is necessary to interpret this data.

This paper sees one's way to serve as substitute for these tables.

The results agree with the table data.

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Vinification par macération carbonique VIII. — Préchauffage de la vendange

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avec la collaboration technique de
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Résumé

Une température élevée (30-35 °C) durant la première phase d'une vinification par macération carbonique est nécessaire pour obtenir des vins harmonieusement constitués. L'obtention de ces températures devient difficile lorsque la vendange est froide à l'encuvage.

Nous avons étudié un système de réchauffage de la vendange non foulée par un contact prolongé avec du moût exogène chaud. Après macération carbonique de cette vendange, les caractéristiques analytiques des vins obtenus different légèrement de celles de vins issus de macération carbonique stricte. Organoleptiquement, les vins du premier type ne sont pas jugés inférieurs aux témoins.

Des études complémentaires sont nécessaires afin de préciser le seuil de température qui doit être atteint par le chauffage.

Mots clés : *macération carbonique, raisin, chauffage.*

Introduction

En vinification par macération carbonique, le niveau de température atteint par la vendange durant la première phase de fermentation détermine pour une grande part les caractéristiques organoleptiques et analytiques du vin obtenu.

Une température de l'ordre de 15 °C appliquée pendant 15 à 20 jours permettra d'élaborer un produit présentant une certaine finesse aromatique. Cet avantage est cependant fugace; il s'accompagne d'un manque de corps et de cou-

leur. Souvent, les vins ainsi élaborés restent élégants et agréables seulement pendant les tous premiers mois qui suivent leur stabilisation biologique (FLANZY et ANDRE, 1973.)

L'obtention de vins rouges de garde, avec des durées de cuvage de 7 à 10 jours, nécessite des températures relativement élevées (25 ° à 35 °C) pour que le métabolisme anaérobie se manifeste pleinement. La température optimale apparente de ce métabolisme se situe, dans des essais de laboratoire, aux alentours de 33 °C (FLANZY, 1978).

De même, dans la vinification en blanc ou en rosé par macération carbonique, il semble préférable d'assurer une haute température de la vendange pendant un temps bref (24 à 36 heures) plutôt que de maintenir les raisins à température réduite durant plusieurs jours. Avec le premier protocole, l'apport aromatique dû au métabolisme anaérobie se manifeste significativement avant que la diffusion des polyphénols et que le catabolisme de l'acide malique ne soient trop importants (FLANZY et ANDRE, 1973).

Différentes techniques ou artifices sont utilisés pour relever la température des raisins en début de cuvaison : chauffage et recyclage du jus accumulé au fond du récipient vinaire; pieds de cuve en pleine activité levurienne; isolation thermique des parois ou circulation d'eau chaude entre les doubles parois du conteneur; localisation de la macération carbonique au milieu d'un ilot de cuves chaudes, en cours de fermentation; choix des heures de cueillette...

Quel que soit le procédé employé, le gain de température et la durée nécessaire pour l'obtenir sont souvent peu satisfaisants. Les transferts thermiques dans des masses d'homogénéité variable comme l'est une vendange entière avec un gaz pour fluide ambiant sont vraisemblablement peu rapides.

Actuellement, l'équipement de certaines caves en matériel de thermotraitement de la vendange offre la possibilité de relever la température des raisins entiers par immersion dans du moût exogène préalablement chauffé.

Durant trois campagnes (1975, 1976, 1977) nous avons réalisé des essais mettant en œuvre ce système au niveau de la cave expérimentale.

Méthodes et techniques

Matériel végétal

La vendange, saine ou légèrement altérée, est formée essentiellement de *Carignan noir* récolté au Domaine expérimental de Pech-Rouge (INRA). Les lots à traiter et les lots témoins sont échantillonnés selon la technique de BENARD et JOURET (1963).

Le moût servant au transfert de chaleur provient du cépage *Clairette blanche* ou du mélange de cépages *Clairette* + *Maccabeu* issus du même vignoble (tabl. 1).

Protocole expérimental

Après stockage d'une nuit à l'extérieur de la cave, pour abaisser la température de la vendange, cette dernière est encuvée dans des récipients de 50 hl ou de 110 hl (tabl. 1). A la fin du remplissage le moût de goutte provenant de l'éclatement des baies lors de la chute ou du tassement de la vendange est retiré de la cuve.

TABLEAU I

Caractéristiques des vinifications

T = lot témoin vinifié en macération carbonique. Ch = lot vinifié en macération carbonique après chauffage de la vendange.
X = moût de goutte en fin de remplissage de la cuve; retiré de la cuve. (Réintroduit après chauffage de la vendange).
Y_1 = jus de chauffage introduit dans la cuve. Y_2 = jus de chauffage retiré de la cuve après macération avec la vendange.
V = volume (l); θ = température (°C); *t* = durée (mn); *d* = densité; AT = acidité totale (g/l H_2SO_4); Mal = acide malique (Meq/l) 520 — 420 — 280 = DO 520 nm, 420 nm, 280 nm.
Pol = polyphénols totaux (g/l acide gallique). Ant = anthocyanes (g/l).
K, Na, Ca = potassium, sodium, calcium (meq/l).
durée 1ere phase F = durée (jours) de la première phase de fermentation.
G, P = volume jus de goutte et jus de presse (l).

$$\rho = \frac{\text{K/3 vendange}}{\text{hl (G + P)}}$$

		1975		1976		1977	
		T	Ch	T	Ch	T	Ch
Cépage expérimenté		Carignan noir (75 %) Grenache blanc (25 % sain		Carignan noir; légèrement altéré		Carignan noir; sain	
Poids vendange (kg)		4 400	4 250	4 390	4 370	6 640	6 640
Température vendange (°C)			22		19		17
Volume cuve (hl)		50	50	50	50	110	110
X	V		360		400		450
	θ		22		22		17
	t écoulement		6		6		20
	d - AT - Mal	1,080 - 5,9				1,090 - 5,20 - 52,2	
	520 - 420 - 280	0,178 - 0,045 - 0,480				0,067- -0,036 - 0,027	
	Pol - Ant					0,620 — 0,070	
	K - Na - Ca	43 - 0,6 - 10,8					
Y_1	Cépage	Clairette		Clairette		Clairette (50 %) + Maccabeu (50 %)	
	V	1900		1250		3630	
	θ	48		52		55	
	t injection	12		11			
	t'	60		40		55	
	d - AT - Mal	1,087 - 4				1,037 - 4,3 - 44,8	
	520 - 420 - 280	0,110 - 0,063 - 0,750				0,070 - 0,055 - 0,750	
	Pol - Ant.					1,500 — 0,063	
	K - Na - Ca	58,4 - 2 - 10					
Y_2	V	1900		1250		3630	
	θ	26					
	t écoulement	10		5			
	d - AT - Mal	1,085 - 4				1,087 - 4,5 - 45,5	
	520 - 420 - 280	0,095 - 0,065 - 0,700				0,080 - 0,050 - 0,620	
	Pol - Ant.					1,480 — - 0,066	
	K - Na - Ca	57,6 - 1,8 - 10,5					
	durée 1ere phase	10	10	10	10	10	9
	C	2 250	2 200	2 550	2 500	2 819	3 416
	P	1 200	1 125	1 000	950	2 346	1 832
	G. 100	65,2	66,2	72	72	54,6	65,1
	G + P	127,5	127,3	123,7	126,7	128,6	126,5
	Marc (kg)	511	472	451	410	831	855

Le jus exogène est extrait par pressurage de la vendange de Clairette préalablement portée à 75 °C dans un appareil de thermotraitement. Ramené aux environs de 50 °C, le jus est soit injecté par le robinet inférieur de la cuve, (1975) soit déversé en jet brisé sur le chapeau de vendange (1976, 1977). L'apport de jus est maintenu jusqu'à submersion constante de la totalité de la vendange. Après une heure de contact « liquide chaud-vendange », un volume de jus équivalent au volume apporté est retiré du récipient vinaire par gravité.

Le moût de goutte initial est réinjecté dans la cuve dont l'atmosphère est alors renouvelée par du dioxyde de carbone jusqu'à saturation.

Le décuvage et le pressurage sont effectués une dizaine de jours après. Le lot témoin est vinifié directement par macération carbonique sans préchauffage de la vendange.

Mesures et analyses

Les mesures de températures sont réalisées par des thermocouples (6) implantés dans des baies de raisin. Les thermocouples sont reliés par câble à un enregistreur externe. Les «baies étalon » sont localisées à différents niveaux de la cuve.

Des sondes thermiques permettent de suivre l'évolution des températures du moût et de la vendange au cours du préchauffage et des deux phases de fermentation.

Les analyses physico-chimiques sont effectuées selon les techniques officielles (Journal officiel, 1963). Les résultats de dégustations sont interprétés par le test de Kramer pour une probabilité de 5 p. 100 (TOMASSONE et FLANZY, 1978).

Résultats

Évolution de la température des raisins et du jus durant le préchauffage

Durant l'apport de moût exogène, la zone d'injection est très chaude. La chaleur diffuse de cette zone vers d'autres régions en suivant des circuits préférentiels à travers la masse encuvée. D'autre part, le moût apporté se refroidit d'autant plus qu'il traverse un volume plus important de vendange. Ainsi, dans le cas d'un arrosage par le sommet de la cuve (1976) le jus situé à ce niveau est, en fin de chambrage (50 mn), à la température de 43 °C alors que le moût du bas de récipient est à 22 °C.

La répartition des températures n'est donc pas homogène durant toute la période de contact avec le moût exogène.

Après retrait de ce dernier, les températures tendent à se régulariser par niveau décroissant de haut en bas du récipient vinaire. Cependant, des différences importantes existent parfois entre les températures observées à différents endroits d'un même niveau (tabl. 2).

A la longue, les écarts initiaux de température entre deux zones diminuent. Les températures enregistrées en divers points de la vendange se regroupent vers des valeurs plus élevées que celle mesurée au départ dans les raisins.

TABLEAU 2

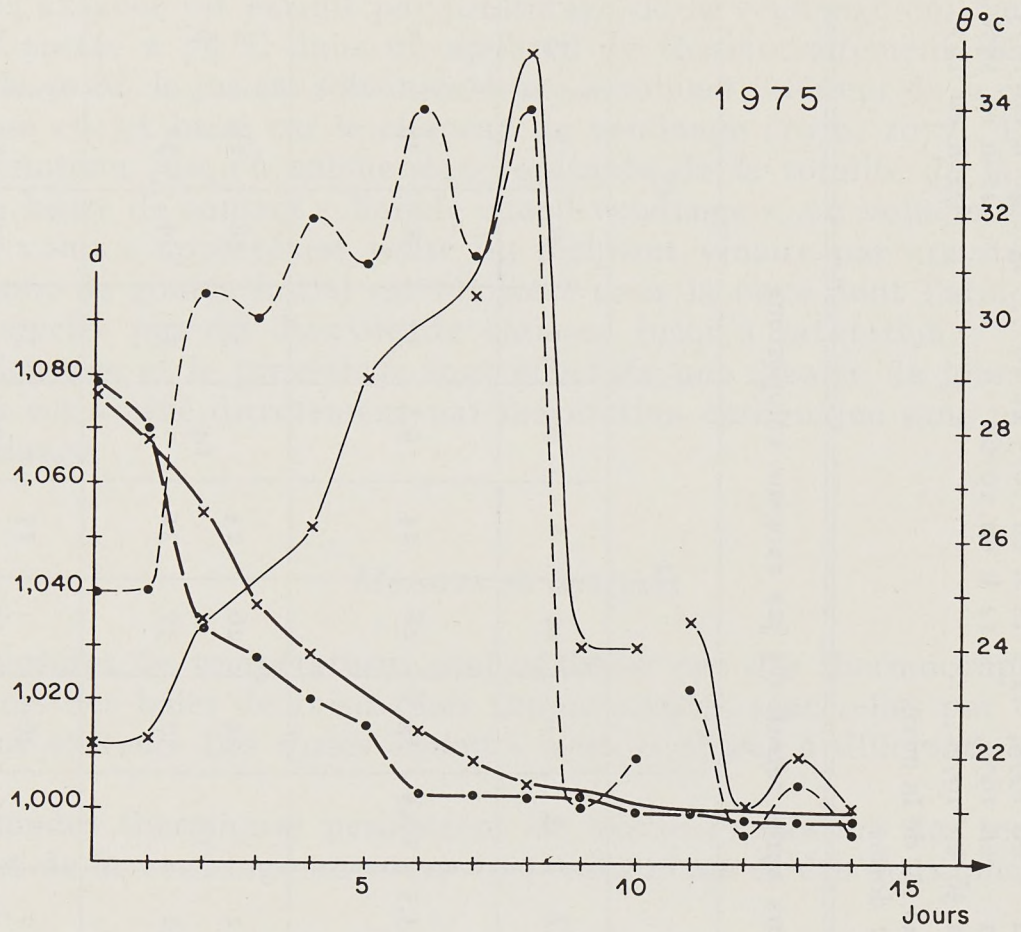
Évolution de la température des raisins

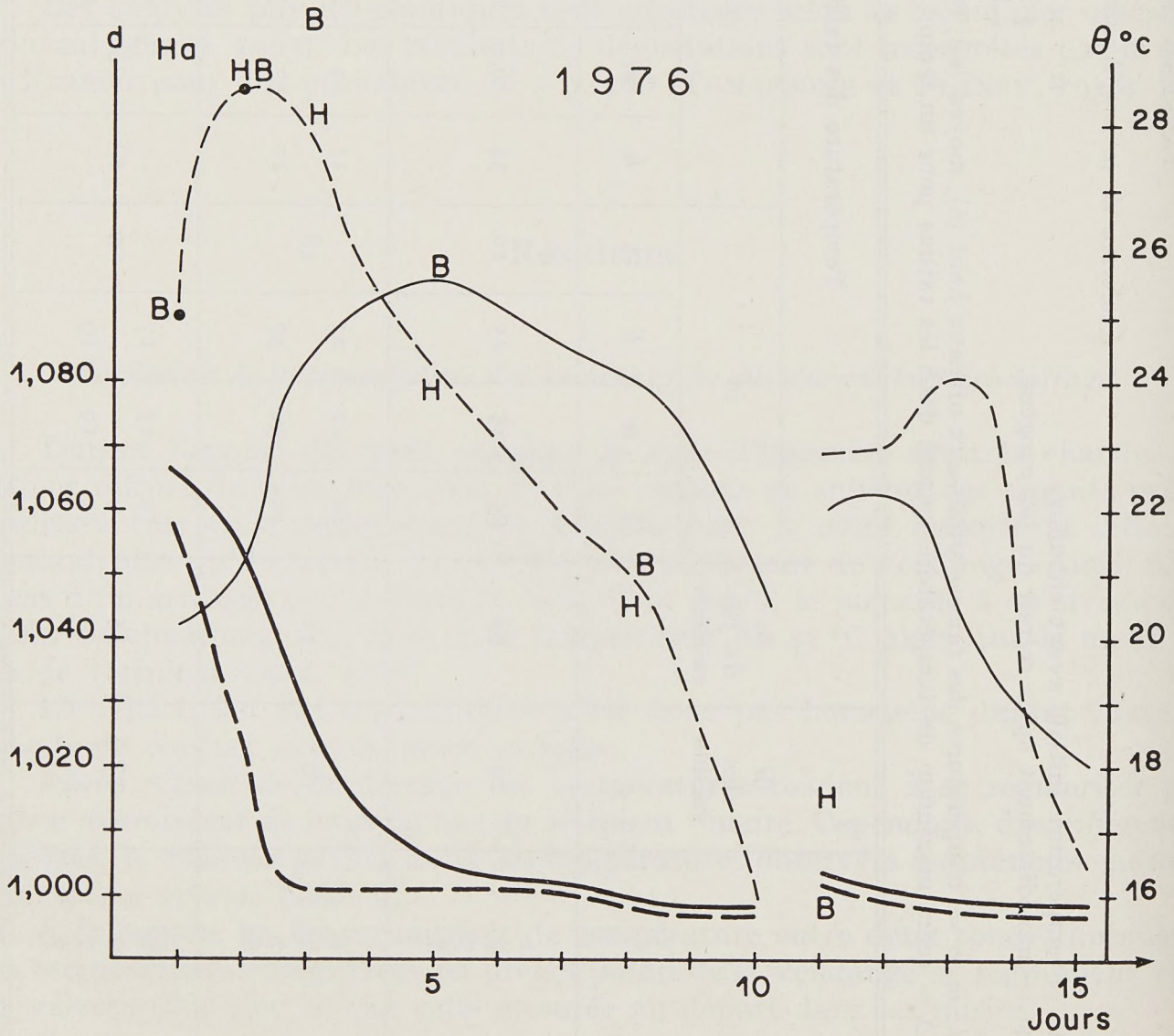
θi = température initiale avant chauffage.
to = immédiatement après retrait du jus exogène.

*t*1 : 3 h en 1975, 2 h en 1976, 1 h 30 en 1977.
*t*2 : 24 h en 1975 et 1977, 12 h en 1976.
*t*3 : 40 h en 1975, 22 h en 1976, 72 h en 1977.

h, *m*. *b* = températures des raisins situés aux niveaux haut (*h*), moyen (*m*), bas (*b*) de la cuve.
Δ = écart maximum de température mesuré dans les raisins pour un temps *t* donné.

	θi des raisins	θi du jus exogène	Température des raisins après retrait du jus exogène caloporteur															
			to				*t*1				*t*2				*t*3			
			h	*m*	*b*	Δ	*h*	*m*	*b*	Δ	*h*	*m*	*b*	Δ	*h*	*m*	*b*	Δ
19 75	22	48	35	25	25	10	34	26,5	23,5	10,5	33,5	28	24	9,5	32	30	25	7
19 76	19	52	30 40	22 36	22 37	18	31 40	24 37	22 32	18	34 37	26 37	23 32	14	32 34	28 34	28 32	6
19 77	17	55	19 42	17 19	17 19	25	42	23	17 19	25	36 42	22	17 19	25	32 41	32	19	22


1975
d
θ°c
1,080
1,060
1,040
1,020
1,000
34
32
30
28
26
24
22
5
10
15
Jours


1976
d
θ°c
Ha
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H
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B
H
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H
1,080
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Jours

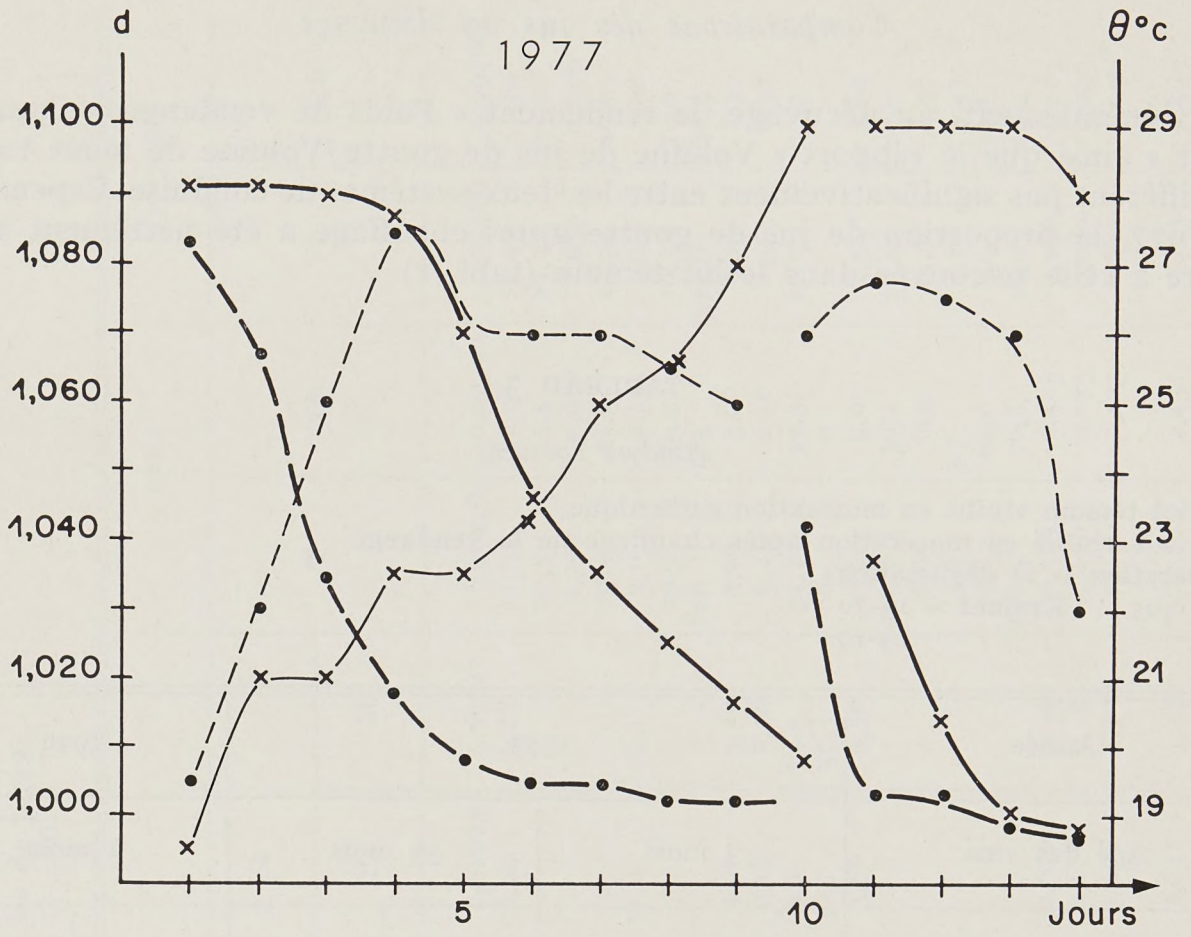

FIG. 1. — *Évolution des densités et des températures des moûts et des vendanges*

——— densité
——— température } du moût témoin.
— — densité
— — température } du moût après préchauffage de la vendange.
. B température de la vendange au niveau haut (H) ou bas (B)
. H de la cuve. } essai 1976.
D : fin de la première phase de fermentation; décuvage.

La température du jus exogène au moment de l'égouttage est très abaissée par rapport à celle notée durant l'injection. En revanche, sa composition varie peu après une heure de macération (tabl. 1).

Caractéristiques des fermentations

La fermentation du moût de goutte provenant d'une vendange préchauffée est accélérée au début de la première phase de fermentation. Une densité voisine de 1 000 est atteinte, par le lot préchauffé, 3 à 4 jours avant, le lot témoin (fig. 1).

La fin de la fermentation alcoolique est à peu près simultanée pour les deux lots.

La fermentation malolactique est plus rapide à la suite du préchauffage (5 jours d'écart) dans l'expérimentation 1977.

L'accélération initiale de la fermentation conduit à une élévation rapide de la température du moût. La somme de températures accumulées dans le jus de goutte de vendange préchauffée est très supérieure dans les premiers jours de fermentation à celle calculée pour le moût de la cuve témoin.

Il en est de même pour les températures enregistrées dans la vendange (fig. 1, 1976).

Comparaisons des jus au décuvage

Généralement, au décuvage, le rendement « Poids de vendange/Volume de moût » ainsi que le rapport « Volume de jus de goutte/Volume de moût total » ne diffèrent pas significativement entre les deux systèmes de conduite. Cependant, en 1977, la proportion de jus de goutte après chauffage a été nettement supérieure à celle retrouvée dans le lot témoin (tabl. 1).

TABLEAU 3

Analyse des vins

T = lot témoin vinifié en macération carbonique
Ch = lot vinifié en macération après chauffage de la vendange
Dégustation = 11 dégustateurs
P = 0,05 Kramer = 13-20
14-19

Année	1975				1976	
Age des vins	3 mois		53 mois		2 mois	
Lot	T	Ch	T	Ch	T	Ch
Densité 20/20	0,99456	0,99486	0,99386	0,99406	0,99589	0,99599
Degré à 20 °C	10,80	10,66	10,33	20,27	9,87	10,11
Extrait sec (g/l)	22,9	23,5	19,8	20,1	23,5	24,8
Cendres (g/l)	2,450	2,450	2,150	2,200	2,600	2,700
Alcalinité des cendres (méq/l)	29,5	28,0	24,0	25,0	31,0	31,0
Sucres (g/l)	1,31	1,23	1,76	1,68	1,70	1,70
Glycérol (g/l)	7,82	8,46				
Aldéhydes (mg/l)	13,2	14,0	22,8	21,1	15,8	22,8
Azote total (mg/l)	126	119	95,2	100,8	133	203
Acidité totale (H_2SO_4 g/l)	3,30	3,30	3,10	3,10	3,70	3,60
Acidité volatile (H_2SO_4 g/l)	0,23	0,27	0,22	0,23	0,50	0,42
Acide tartrique (méq/l)	30,0	32,0	25,3	25,3	32,0	31,3
Acide malique (méq/l)	4,2	5,0			0	0
pH	3,58	3,60	3,55	3,50	3,53	3,60
Fermentation malolactique	Faite	Faite			Faite	Faite
K (méq/l)	28,2	28,2	26,1	26,1	27,4	28,7
Na (méq/l)	0,26	0,26	0,26	0,34	0,87	0,78
Ca (méq/l)	3,7	3,4	4,9	4,85	4,5	4,4
SO_2 libre (mg/l)	32,0	32,0	16,0	12,8	51,2	51,2
SO_2 total (mg/l)	44,8	38,4	25,6	19,2	57,6	57,6
Matières colorantes (D.O 520 nm)	0,305	0,305	0,070	0,070	0,294	0,282
Matières colorantes (D.O 420 nm)	0,090	0,088	0,043	0,044	0,083	0,124
Matières tanoïdes (D.O 280)	0,715	0,760	0,620	0,650	0,640	0,695
Polyphénols totaux (g/l acide gallique)			1,630	1,744	1,140	1,288
Anthocyanes (g/l)			0,087	0,061	0,353	0,320
Dégustation			20	13		

TABLEAU 4

Analyse des moûts et des vins de l'essai 1977

T = lot témoin vinifié en macération carbonique.
Ch = lot vinifié en macération après chauffage de la vendange.
Dégustation = 11 dégustateurs. P = 0,05, Kramer = 13-20; 14-19.

G = jus de goutte.
P = jus de presse.
M = G + P.

Age des vins	Décuvage						3 mois		30 mois	
Lot	T			Ch			T	Ch	T	Ch
Type	G	P	M	G	P	M				
Densité 20/20	1,00489	1,04173	1,01972	1,0007	1,02665	1,00841	0,99416	0,99496	0,99386	0,99466
Degré à 20 °C	9,28	6,86	8,44	9,99	8,94	9,28	11,62	11,28	11,47	11,39
Extrait sec (g/l)							22,5	25,5	23,2	25,0
Cendres (g/l)							2,750	2,900	2,650	2,600
Alcalinité des cendres (méq/l)							29,5	21,0	25,75	26,0
Sucres (g/l)	14,2	105	51,5	3,60	71,0	17,1	2,0	2,10	2,05	2,08
Aldéhydes (mg/l)							29,9	19,3	19,3	14,0
Azote total (mg/l)							126	163	117,6	162,4
Acidité totale (H_2SO_4 g/l)	5,40	5,30	5,20	5,60	5,00	5,20	3,60	3,60	3,20	3,30
Acidité volatile (H_2SO_4 g/l)	0,11	0,11	0,13	0,14	0,18	0,18	0,24	0,30	0,30	0,38
Acide tartrique (méq/l)	66,7	66,7	69,3	65,1	63,2	64,0	32,7	33,3	26,0	26,7
Acide malique (méq/l)	38,8	39,6	38,8	39,6	36,7	37,3	0	0		
pH	3,50	3,42	3,48	3,50	3,50	3,50	3,60	3,65	3,58	3,60
K (méq/l)							29,7	30,2	27,4	27,7
Na (méq/l)							6,95	6,52	6,78	6,38
Ca (méq/l)							3,6	3,7	4,35	4,45
SO_2 libre (mg/l)							70,8	64,0	9,6	12,8
SO_2 total (mg/l)							102,4	76,8	16,0	19,2
Matières colorantes D.O 520 nm	0,700	0,660	0,700	0,660	0,610	0,650	0,515	0,485	0,196	0,217
Matières colorantes D.O 420 nm	0,165	0,170	0,167	0,150	0,175	0,155	0,170	0,175	0,087	0,090
Matières colorantes D.O 280 nm	1,240	1,160	1,220	1,300	1,200	1,300	1,040	1,105	0,880	1,000
Polyphénols totaux (g/l acide gallique)	2,223	2,679	2,690	2,625	3,021	3,249	2,188	2,451	2,223	2,565
Anthocyanes (g/l)	1,037	0,923	0,967	1,011	0,941	0,879	0,685	0,676	0,140	0,175
Dégustation									16	17

Dans l'essai 1977, le préchauffage entraîne des modifications dans la composition des moûts de goutte et de presse au décuvage : densité et teneur en sucres plus basses, quantités d'éthanol plus élevées que dans le lot témoin. La concentration en malate du jus de presse, en anthocyanes et l'indice DO 520 nm sont inférieurs aux critères correspondants du témoin alors que les indices de polyphénols et DO 280 nm sont supérieurs.

Analyses des vins

Les analyses après stabilisation biologique des vins (fermentation malolactique terminée) et après maturation de 30 (1977) ou 53 mois (1975) conduisent aux observations suivantes (tabl. 3 et 4).

Les vins issus de vendange préchauffée ont des valeurs d'extrait sec, d'indices de polyphénols et de DO 280 nm plus élevées que celles des vins témoins. Leur densité et leur teneur en azote ont tendance à être également supérieures. En revanche, les indices « DO 520 nm + 420 nm » et DO 520 nm /DO 280 nm » ainsi que parfois le degré alcoolique sont inférieurs après chauffage de la vendange.

A l'issue de 53 mois de conservation, le vin de vendange préchauffée de la récolte 1975 est, organoleptiquement, préféré au vin correspondant de vendange « froide ».

Discussion

Les résultats obtenus à partir des trois couples d'essais permettent-ils d'apprécier l'intérêt du système utilisé pour préchauffer la vendage en macération carbonique?

Les différences analytiques entre vins d'un même couple sont généralement réduites pour la plupart des critères étudiés. D'autre part, les vins issus de vendange préchauffée n'ont pas été jugés inférieurs sur le plan organoleptique aux vins témoins. Au contraire, après une garde de longue durée (53 mois) l'échantillon « chauffé » a été préféré; la dégustation n'a porté, cependant, que sur un seul couple de vins.

Le traitement appliqué à la vendange n'a donc pas influencé défavorablement les caractéristiques du produit final.

Il est vraisemblable, en outre, que les aspects positifs du chauffage n'aient pu être nettement mis en évidence. En effet, les écarts de température entre échantillons correspondants d'un même essai, écarts importants dans les premiers jours se sont fortement atténués par la suite. Les températures maximales atteintes et les sommes de température accumulées par chacun des lots sont très proches. La montée en température des lots témoins a été suffisante pour compenser, au moins partiellement, les effets d'une température initialement élevée dans les lots correspondants. Nous admettons dans ce raisonnement que les températures enregistrées dans le moût traduisent assez fidèlement les températures réelles de la vendange.

Les différences observées dans nos essais devraient donc être plus accentuées pour des durées raccourcies de la première phase de fermentation ou pour des températures très basses de la matière première.

L'augmentation importante de la vitesse de fermentation du moût dans les premiers jours de macération après chauffage de la vendange, est un facteur favorable au bon déroulement de la vinification. En effet, des départs en fermentation lents peuvent faciliter le développement de microorganismes indésirables et être, ainsi, des sources d'accidents (Flanzy et André, 1973).

Le préchauffage de la vendange entraîne, cependant, des conséquences plus ou moins souhaitables.

La forte accumulation de la chaleur en peu de jours risque de conduire à des déperditions importantes de substances volatiles élaborées par les divers métabolismes mis en jeu et participant à l'arôme du vin.

La proportion du jus de goutte « chauffé » augmente par rapport à celle du témoin lorsque la température de ce dernier est relativement basse et lorsque les quantités de vendanges mises en œuvre sont importantes (1977).

La composition des jus au décuvage est modifiée. Le jus de presse, en particulier, est moins riche en sucres et sa teneur en malate est légèrement plus faible que celle du témoin.

Un accroissement de densité, d'extrait sec, d'azote total, des indices polyphénoliques apparaît dans les vins de préchauffage.

Cet ensemble d'observations tend à montrer certaines analogies entre la vinification par vendange foulée et la macération carbonique lorsque celle-ci est précédée de l'immersion de la vendange dans du moût chaud.

Un tel système correspond à un compromis entre une macération carbonique stricte où l'intégralité du maximum des baies est recherchée et une macération carbonique avec une fraction importante de la vendange formées de baies éclatées comme cela se produit dans certaines conditions : géométrie des cuves, remontages (André *et al.*, 1967; Benard *et al.*, 1971).

Dans des conditions climatiques sévères, le praticien peut être placé devant un choix : accepter une diminution de la fraction de vendange bénéficiant du métabolisme anaérobie en cherchant à élever la température ou risquer une action limitée de ce métabolisme sur la totalité d'une vendange trop froide.

Il est intéressant de noter que la macération carbonique permet, généralement, d'étaler la transformation des sucres sur l'ensemble des deux phases de fermentation. Elle évite ainsi, durant la période de macération, une augmentation de température aussi élevée qu'en vendange foulée. Cet accroissement conduit parfois à des arrêts de fermentation dans des milieux très riches en sucres (Charnay et André, 1962).

Paradoxalement, le métabolisme anaérobie des raisins ne donne son plein effet, pour des durées normales de cuvaison, qu'avec de fortes températures. En outre, les microorganismes trouvent dans le moût issu de ces raisins un milieu propice à leur développement. Les arrêts de fermentation provoqués par de hautes températures étant très rares en macération carbonique, il convient donc de rechercher une température correcte pour le métabolisme anaérobie.

L'objectif qui doit être poursuivi est l'atteinte d'un seuil de température à partir duquel le réchauffement de la vendange est autocatalytique par activation du métabolisme anaérobie et de la fermentation levurienne du moût en fond de cuve. Ce seuil est difficile à déterminer d'une façon précise. Toutefois, à partir d'observations réalisées sur plusieurs années, il semble que lorsque la température de 22 °C à 25 °C est atteinte rapidement dans la masse, le processus catalytique se déclenche sans tarder.

L'immersion de la vendange dans du moût chaud est l'un des systèmes pouvant être utilisé pour atteindre le seuil de température requis.

Des contraintes interviennent dans le choix de la température à laquelle doit être porté le moût caloporteur et la durée de son action. Il convient, à la fois, d'éviter l'exaltation des activités oxydasiques et de ne pas inhiber les enzymes du métabolisme anaérobie. Les échanges de constituants entre moût exogène et vendange doivent être limités.

Le système mécanique d'apport du fluide chaud dans la vendange peut conditionner la répartition de la chaleur dans la cuve. Nos essais ont montré que l'injection du moût par le bas de la cuve engendrait la formation de veines liquides.

L'arrosage du chapeau de la vendange semble améliorer l'homogénéisation de la distribution des températures mais risque de provoquer des éclatements de baies si le jet de moût n'est pas suffisamment brisé.

Le chauffage de la vendange avant son encuvage a été également envisagé au moyen de jets de vapeur d'eau ou de microondes. Les essais entrepris en 1973 avec la vapeur doivent être repris tant au Laboratoire qu'en Cave Pilote. La mesure du gradient de température dans les baies et dans le fluide ambiant devrait permettre une meilleure maîtrise des conditions d'élaboration des vins par macération carbonique.

Conclusion

Le réchauffage d'une vendange non foulée par du moût exogène préalablement porté à température élevée peut se réaliser relativement aisément. Ainsi, la submersion d'une vendange froide (17 à 19 °C) de Carignan dans du moût de Clairette porté à 55 °C permet, au bout d'une heure, de relever la température moyenne des raisins aux alentours de 30 °C. Cependant, la répartition des températures des baies dans la cuve est hétérogène et l'homogénéisation thermique n'intervient qu'après un ou deux jours.

Durant l'anaérobiose créée à la suite du préchauffage, les phénomènes fermentaires sont accélérés par rapport à ceux d'une vinification par macération carbonique de vendange froide. La stabilisation biologique des vins intervient à peu près simultanément pour les deux types de produits. Si le préchauffage par contact avec le liquide caloporteur modifie les valeurs de certains critères analytiques des vins, il ne semble pas altérer leurs caractères organoleptiques.

Inutile dans le cas de raisins récoltés par temps chaud, ce système peut-être précieux pour traiter des vendanges froides par macération carbonique. Cependant, un tel procédé est à utiliser avec prudence dans la mesure où il doit favoriser le métabolisme anaérobie des raisins et non conduire à une vinification très proche de celle d'une vendange foulée.

Sauf dans le cas de vinification en rosé et en blanc, il n'est vraisemblablement pas nécessaire d'obtenir la température optimale de vinification par le seul chauffage artificiel. Il semble plus raisonnable d'atteindre un seuil de température à partir duquel le métabolisme anaérobie s'accélèrera et atteindra naturellement son régime maximal.

La détermination de ce seuil devrait faire l'objet de prochains travaux.

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Summary

Wine making by carbonic maceration. VIII. Preheating of grapes

A high temperature (30-35 °C) during the first stage of fermentation by carbonic maceration is necessary to obtain harmonious wines. It is difficult to get these temperatures when the grapes are cold, at grape ratting.

We have studied a warming design for no crushed grape by long contact with hot exogenous must. After the carbonic maceration of those grapes, the analytical wine characteristics ere slightly different from those coming from strict carbonic maceration. From organoleptic view point, the quality of the first type wines are comparable to the control.

Complementary studies are necessary to definite the temperature value that must be obtained by heating.

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Évolution de la teneur en carotenoïdes de la tomate en fonction des stades de maturation et des conditions culturales

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Résumé

Le travail a porté sur l'évolution au cours de la maturation des fruits, de la teneur en caroténoïdes d'une variété de tomate, H63-5. Les influences respectives du mode de culture (sous serre en verre, tunnel plastique et plein champ) et de la place du fruit sur la plante ont été étudiées. Nous confirmons l'aptitude du lycopène et du carotène à servir de critères permettant de différencier des stades physiologiques de la tomate. En effet, l'évolution de ces caroténoïdes n'est pas influencée par les facteurs considérés; seules les teneurs diffèrent.

Mots clés : *Caroténoïdes, tomate, maturation, méthodes de culture, critères de maturité.*

I. — Introduction

En 1975, une étude a été entreprise sur la conservation en frais de la tomate (Cabibel, Ferry, 1975). Des essais préliminaires ont montré que la maîtrise de la conservation des fruits frais nécessite la connaissance de leur stade de maturité au moment de la cueillette. Dans ce but, nous avons recherché et étudié certains critères caractéristiques des différents moments de la vie de la tomate.

Une première étape a consisté à évaluer la précision de la mesure de quelques caractéristiques physicochimiques de la tomate : fermeté, extrait sec, substances insolubles dans l'alcool (AIS), sucres réducteurs, β carotène lycopène et chlorophylle sur un échantillonnage compatible avec nos possibilités expérimentales (Cabibel, Ferry, 1979).

En plus de l'AIS et de la fermeté, nous avons retenu le lycopène qui, malgré la mauvaise précision obtenue, présente des variations significatives d'un stade de maturité à l'autre. Il en est de même pour le β carotène.

La deuxième étape a eu pour but de déterminer l'influence, d'une part des modes de culture et d'autre part de la position des fruits sur la plante, sur l'évolution des pigments en cours de maturation.

Le présent travail rapporte nos observations sur l'un de ces critères, les caroténoïdes, pour la variété H_{63_5}, cultivée sous serre en verre, sous tunnel plastique et en plein champ.

II. — Méthodes et techniques

2.1. — *Protocole d'essai*

Les tomates proviennent de trois origines :

— serre en verre située à Châteaurenard sur un sol limonoargileux présentant plus de 50 p. 100 d'éléments fins;
— tunnel plastique à Rognonas sur un sol de même type;
— plein champ : champ expérimental de la Station d'Amélioration des plantes maraîchères de Montfavet sur un sol de Costières à pourcentage important de galets et cailloux.

Au moment de la floraison, les fleurs des premier et sixième bouquets ont été étiquetées de façon à repérer à un jour près la date de fécondation.

Pour constituer un échantillon de 90 tomates au même stade de maturité, nous avons été obligés de récolter les fruits en 1 à 4 fois sans dépasser un intervalle total de 10 jours, sur environ 30 plants. Les fruits sont immédiatement entreposés au congélateur à — 20 °C. Chaque échantillon est ensuite réparti en 3 lots de 30 tomates pour permettre la répartition des analyses.

Nous avons considéré les stades suivants :

— Stade vert ou 1 calibre final (« mature green »).
— Stade blanc ou 2.
— Stade jaune ou 3.
— Stade rose ou 4.
— Stade rouge ou 5.

Nous avons déterminé sur chaque lot :

— l'extrait sec,
— la composition en caroténoïdes par chromatographie d'adsorption sur alumine.

2.2. — *Méthodes de dosage*

— Extrait sec : sur 5 g selon la méthode AOAC 1975 n° 22018.
— Dosage des caroténoïdes.

2.21. — *Extraction des caroténoïdes.*

Les fruits sont broyés avant décongélation totale. Les prises d'essai sont de 25 g pour le stade 5,50 g pour le stade 4 et 100 g pour les autres.

Les pigments sont extraits par l'acétone jusqu'à décoloration complète de la

pulpe. Ils sont ensuite transférés dans l'éther de pétrole. Cette solution est mise au contact de 100 ml de méthanol contenant 12 p. 100 de KOH pour la saponification pendant 3 h à la température ambiante (KARRER, JUCKER E., 1950).

La phase inférieure méthanolique est extraite par l'éther éthylique. Après séparation des phases, la solution éthérée est desséchée sous vide à l'évaporateur rotatif à 35 °C. Le résidu sec est repris dans 50 ml d'éthanol 96 °GL.

Cette solution contient les xanthophylles, se réduisant dans notre cas à la lutéine, dont la teneur est directement mesurée par spectrophotométrie à 442 nm.

La phase supérieure éthéropétrolique est lavée trois fois à l'eau, puis séchée sur sulfate de sodium anhydre. Elle est alors prête pour la chromatographie.

2.22. — *Chromatographie d'adsorption sur colonne d'alumine.*

— Nous avons choisi la méthode utilisée par KUSHAWAHA *et al.* (1970). Le support est de l'alumine Merck d'activité I, désactivée partiellement par 1 p. 100 d'eau. L'éluant est un mélange éther de pétrole — éther éthylique contenant des proportions croissantes d'éther éthylique.

Les fractions obtenues sont desséchées à l'évaporateur rotatif sous vide à 35 °C, puis reprises dans un volume connu V d'éther de pétrole. La densité optique est mesurée au spectromètre Beckman DK 2A aux longueurs d'onde précisées dans le tableau 1.

Les teneurs sont ensuite calculées de la façon suivante :

Concentration en µg de pigments pour : $\frac{\text{D.O.} \times V \times 100 \times 100}{100 \times p \times \text{E.S} \times 1 \times E^{\,1\,\%}_{1\ \text{cm}}}$

100 g de poids sec de l'échantillon initial;

avec p = prise d'essai en poids frais

1 = cuve de 1 cm de trajet optique

$E^{\,1\,\%}_{1\ \text{cm}}$ = coefficient d'extinction spécifique du carotène considéré dans le solvant utilisé (GOODWIN T. W., 1955).

E.S. = extrait sec de l'échantillon en g pour 100 g de produit frais.

TABLEAU 1

Longueur d'onde maximum de mesure des carotènes obtenus par élution sur colonne d'alumine

Éluant		Caroténoïde obtenu	λ max. de mesure	$E^{\,1\,\%}_{1\ \text{cm}}$ dans l'éther de pétrole
Ether de Pétrole	Éther Éthylique			
93	7	α-carotène	448	2 710
85	15	β-carotène	450	2 650
70	30	Néoβcarotène B	446	2 650
35	65	Néolycopène A	464	2 260
—	100	Lycopène	471	3 470
		Lutéine	442	2 450 dans l'éthanol

TABLEAU 2

Extrait sec exprimé en grammes pour 100 *g de fruits frais*
(moyenne de 3 répétitions)

	Serre verre			Tunnel plastique			Plein champ		
	Stades de maturité	Dates de récolte	E.S.	Stades de maturité	Dates de récolte	E.S.	Stades de maturité	Dates de récolte	E.S.
1er bouquet	Stade I	3/5, 6/5, 10/5	7,29	Stade I	28/5	6,99	Stade I	25/6	6,79
	Stade II . . .	3/5, 6/5	6,61	Stade II	24/5 28/5 31/5 et 4/6	6,59	Stade II	25/6	6,93
	Stade III . . .	29/4, 3/5, 6/5	6,47	Stade III . . .	24/5, 28/5, 4/6	6,79	Stade III . . .	21, 25 et 29/6	7,01
	Stade IV . . .	29/4, 6/5, 10/5	6,51	Stade IV . . .	28/5, 31/5, 8/6	6,43	Stade IV . . .	24 et 25/6	6,72
	Stade V	29/4, 6/5, 10/5	6,13	Stade V	24 et 28/5	6,95	Stade V	21 et 26/5	6,86
6e bouquet	Stade I	4/6 et 8/6	8,25	Stade I	25/6	7,26	Stade I	23, 30/7 et 5/8	8,34
	Stade II	4,6 et 8,6	7,88	Stade II . . .	25 et 29/6	7,41	Stade II . . .	23 et 30/7	7,75
	Stade III . . .	8,6	8,10	Stade III . . .	25 et 29/6	6,94	Stade III . . .	23 et 30/7 . .	8,08
	Stade IV . . .	4,6 et 8,6	7,90	Stade IV . . .	2/5 et 9/7 . .	6,27	Stade IV . . .	23 et 30/7 . . .	8,10
	Stade V	4/6	7,94	Stade V	2 et 5/7	7,25	Stade V	30/7	8,33

III. — Résultats

Les extraits secs utilisés pour calculer les teneurs en caroténoïdes par rapport au poids sec sont donnés au tableau 2.

Les évolutions des différents caroténoïdes sont présentées sur les figures 1 à 6. Les résultats ont été analysés par la méthode statistique de l'analyse de variance (tabl. 3 à 8).

Les actions simples des trois facteurs, mode de culture, position du bouquet et stade de maturité, ainsi que les interactions de premier ordre sont souvent significatives. De ce fait, l'exposé précis des résultats détaillés serait confus. D'une manière générale nous nous limiterons donc à énoncer les actions simples de chacun de ces facteurs. Les interactions ne seront mentionnées que lorsqu'elles revêtent une certaine importance.

3.1. — *Lutéine* (tabl. 3, fig. 1)

Mode de culture

C'est en plein champ qu'on trouve les teneurs les plus élevées; les deux cultures sous abris, serre verre ou tunnel plastique, ont des teneurs égales.

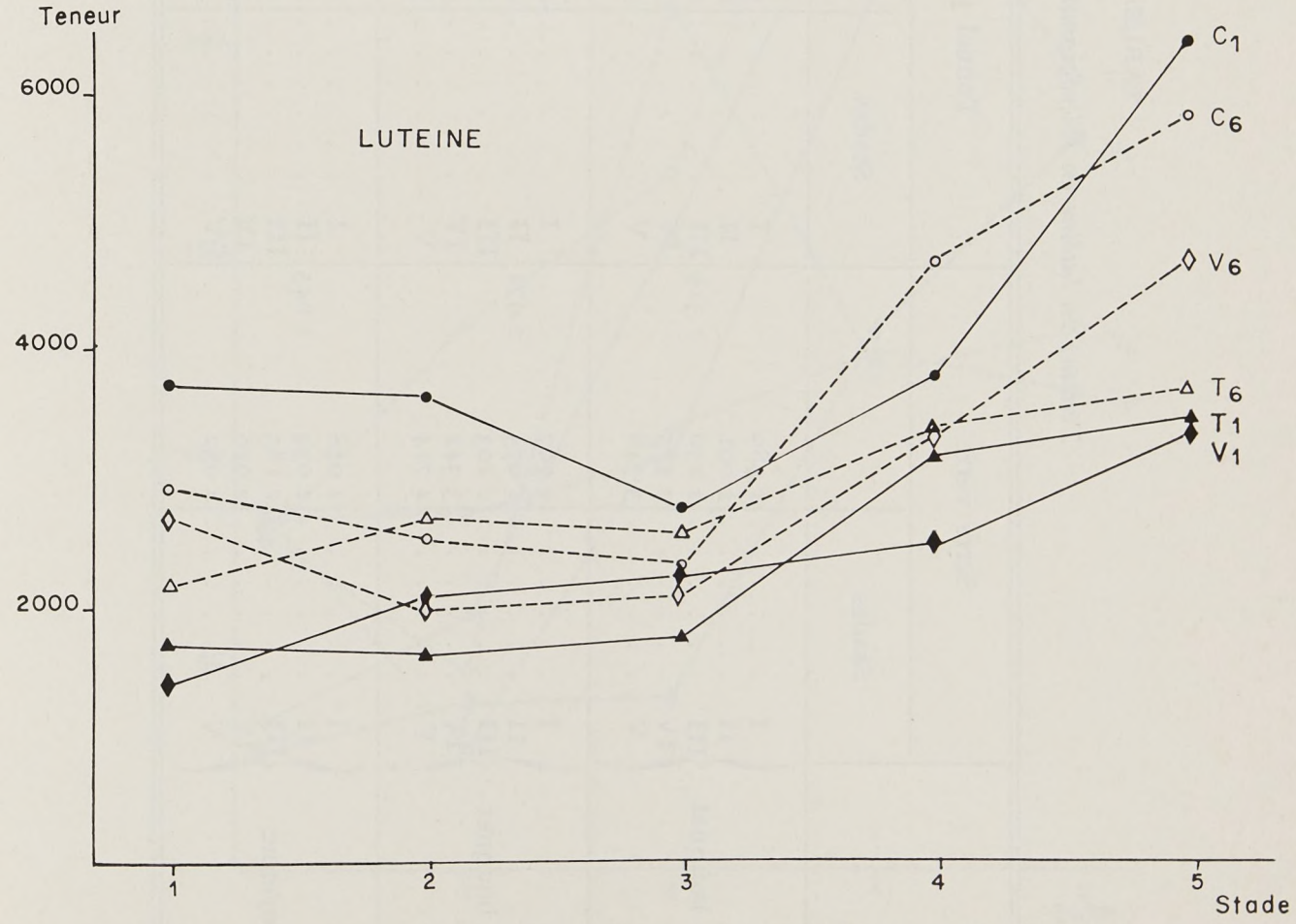

FIG. 1. — *Teneurs en lutéine en fonction de la maturation* (exprimées en microgrammes pour 100 g de matière sèche).

TABLEAU 3

Teneurs en lutéine en microgrammes pour 100 g de matière sèche

		Serre verre		Tunnel plastique		Plein champ		Moyenne	
	Stades			Stades		Stades			
1er bouquet	I	1 389		I 1 724		I 3 736		2 283	
	II	2 091		II 1 661	2 364	II 3 646		2 466	
	III	2 250	2 319	III 1 782		III 2 746	4 059	2 259	2 914
	IV	2 515		IV 3 178		IV 3 791		3 161	
	V	3 348		V 3 477		V 6 375		4 401	
6e bouquet	I	2 707		I 2 177		I 2 922		2 602	
	II	1 975	2 971	II 2 673		II 2 343	3 652	2 398	
	III	2 103		III 2 589	2 894	III 2 339		2 348	3 172
	IV	3 342		IV 3 354		IV 4 663		3 786	
	V	4 714		V 3 675		V 5 790		4 726	
Moyenne	I	2 048		I 1 951		I 3 329		2 443	
	II	2 034	2 645	II 2 167	2 629	II 3 095	3 855	2 432	
	III	2 183		III 2 185		III 2 543		2 304	
	IV	2 928		IV 3 266		IV 4 227		3 474	
	V	4 032		V 3 576		V 6 083		4 564	

Position du bouquet : en plein champ, le premier bouquet a une teneur plus élevée que le sixième. C'est l'inverse pour les cultures sous abri.

Stade de maturité : schématiquement, les teneurs sont égales pour les stades 1, 2 et 3; la teneur augmente au stade 4, puis au stade 5.

3.2. — $\vec{\alpha}$ *carotène* (tabl. 4, fig. 2)

Mode de culture

La teneur la plus faible se trouve dans les cultures faites sous serre verre, la plus forte est en plein champ; sous tunnel plastique, la teneur est intermédiaire et égale à l'une ou à l'autre des précédentes suivant le rang du bouquet et le stade de maturité.

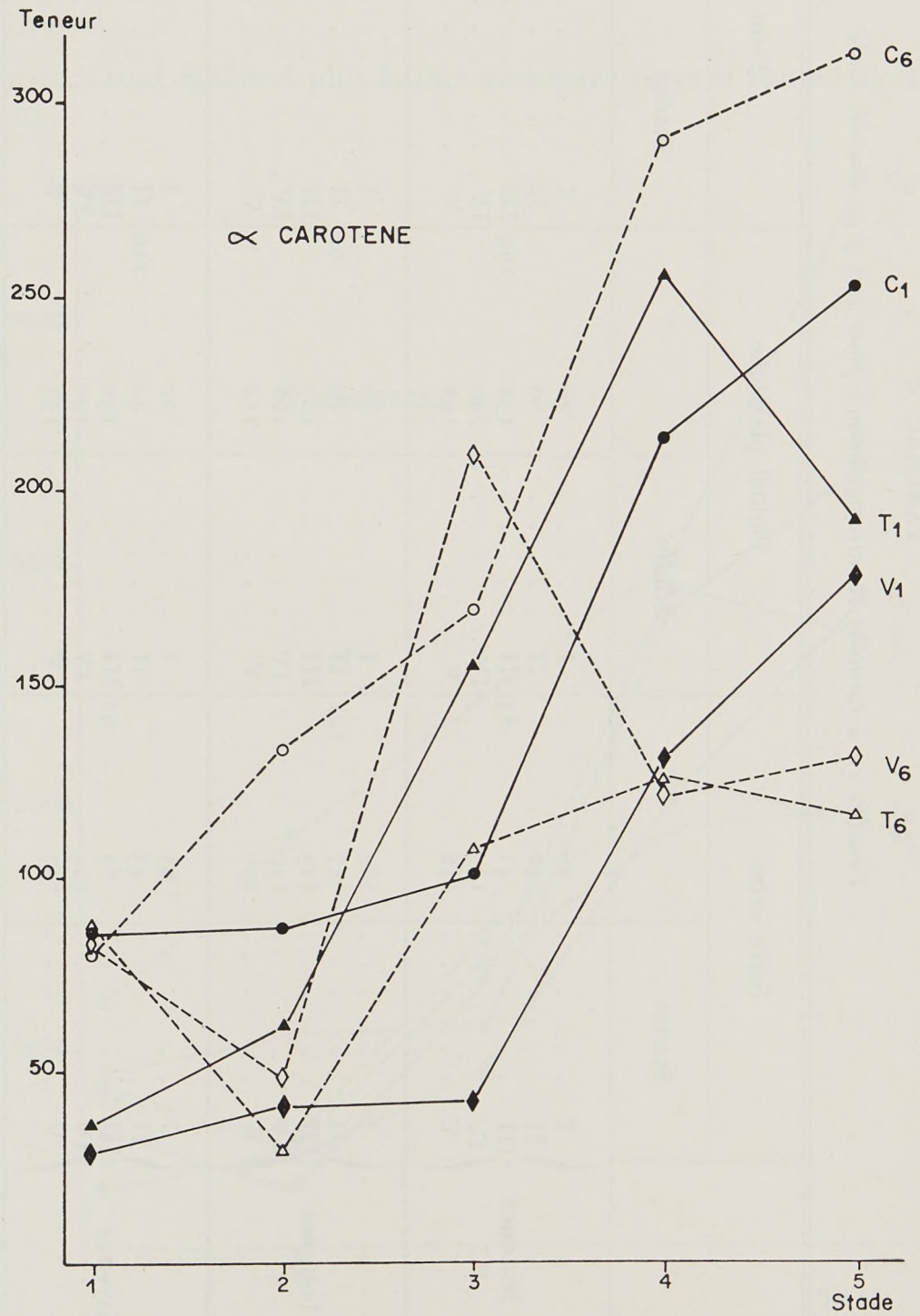

FIG. 2. — *Teneurs en α carotène en fonction de la maturation* (exprimées en microgrammes pour 100 g de matière sèche).

TABLEAU 4

Teneurs en α carotène en microgrammes pour 100 g de matière sèche

		Serre verre		Tunnel plastique		Plein champ		Moyenne	
	Stades			Stades		Stades			
1er bouquet	I	28		I 36		I 85		49	
	II	40		II 60		II 86		62	
	III	41	83	III 154	139	III 100	148	98	123
	IV	130		IV 256		IV 214		200	
	V	178		V 192		V 254		208	
6e bouquet	I	82		I 87		I 81		83	
	II	47	101	II 29	92	II 145		73	
	III	125		III 107		III 167	200	133	131
	IV	119		IV 126		IV 291		179	
	V	130		V 114		V 316		186	
Moyenne	I	55		I 61		I 83		66	
	II	43		II 44	116	II 115	174	68	
	III	83	92	III 130		III 133		115	
	IV	124		IV 191		IV 252		189	
	V	154		V 153		V 284		197	

Position du bouquet : les résultats sont très variables, en fonction du niveau des autres facteurs, la teneur du premier bouquet est inférieure, égale ou supérieure à celle du sixième bouquet.

Stade de maturité

Le stade 1 donne la même teneur que le stade 2, puis la teneur augmente au stade 3 et au stade 4. Le stade 5 donne généralement la même teneur que le stade 4, sauf sous tunnel plastique.

3.3. — β *Carotène* (tabl. 5, fig. 3).

Mode de culture

Les teneurs sont égales et plus faibles sous serre verre et tunnel plastique qu'en plein champ.

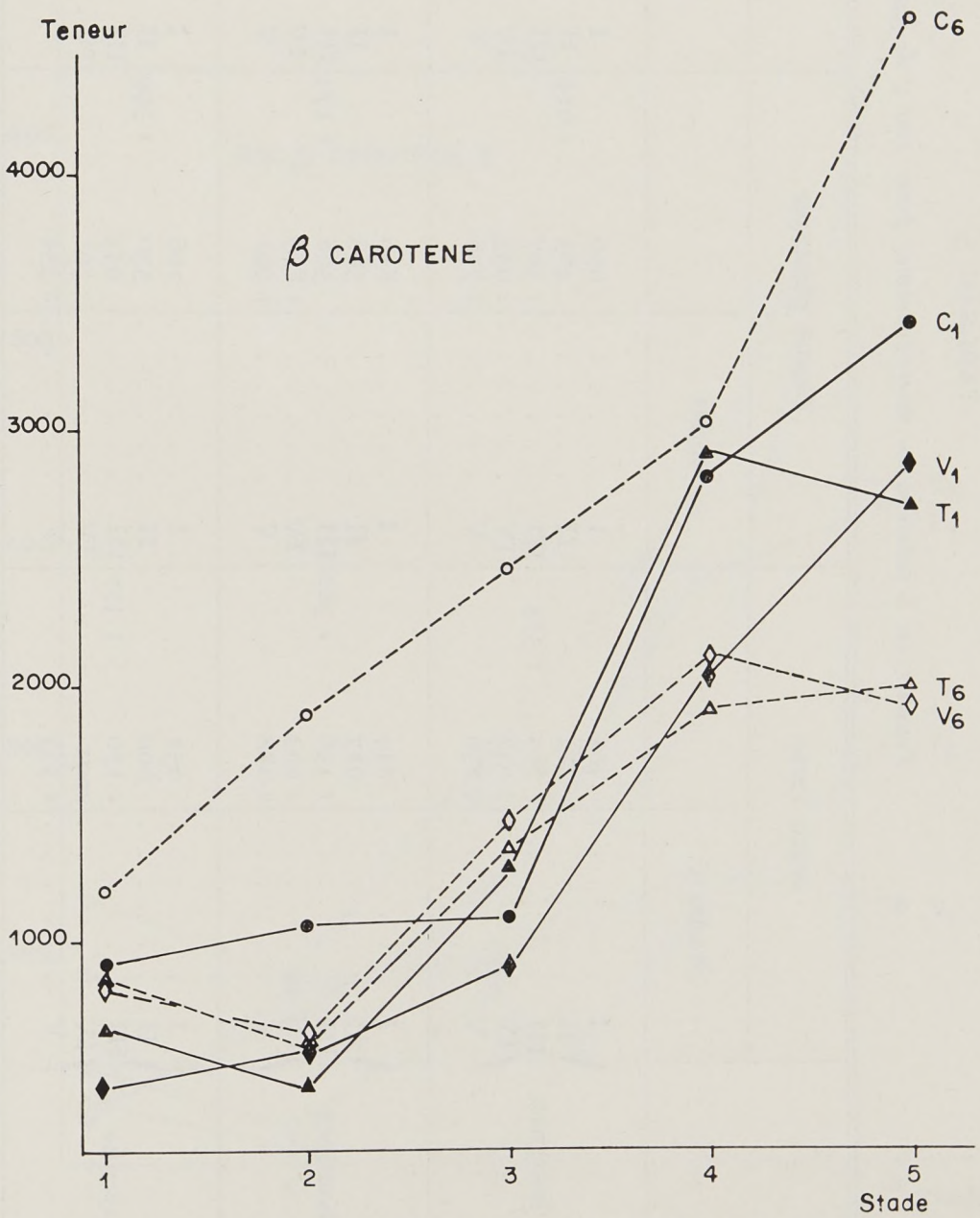

FIG. 3. — *Teneurs en β-carotène en fonction de la maturation* (exprimées en microgrammes pour 100 g de matière sèche).

TABLEAU 5

Teneurs en β carotène en microgrammes pour 100 g de matière sèche

	Stades	Serre verre		Tunnel plastique		Plein champ		Moyenne	
1er bouquet	I	464		I 676		I 887		675	
	II	582		II 463	1 619	II 1 084	1 869	709	
	III	895	1 373	III 1 307		III 1 097		1 099	1 621
	IV	2 048		IV 2 933		IV 2 838		2 606	
	V	2 879		V 2 716		V 3 442		3 012	
6e bouquet	I	844		I 856		I 1 206		969	
	II	617		II 598		II 1 910		1 042	
	III	1 456	1 380	III 598	1 179	III 2 466	2 689	1 507	1 749
	IV	2 093		IV 1 849		IV 3 230		2 391	
	V	1 888		V 1 992		V 4 635		2 848	
Moyenne	I	654		I 766		I 1 047		822	
	II	600		II 530	1 399	II 1 497	2 279	876	
	III	1 176	1 377	III 953		III 1 781		1 303	
	IV	2071		IV 2 391		IV 3 033		2 498	
	V	2 383		V 2 354		V 4 039		2 925	

Position du bouquet

Sous serre verre, il n'y a pas de différence entre le premier et le sixième bouquet. Le sixième bouquet est inférieur au premier sous tunnel plastique. Le sixième bouquet a une teneur plus élevée que le premier bouquet en plein champ.

Stade de maturité

En plein champ, la teneur augmente régulièrement alors qu'en serre verre et tunnel plastique, elle n'augmente qu'à partir du troisième stade.

3.4. — *Néoβcarotène B* (tabl. 6, fig. 4)

Mode de culture

On trouve les teneurs les plus élevées en plein champ. Le tunnel plastique présente des teneurs légèrement plus faibles que la serre en verre.

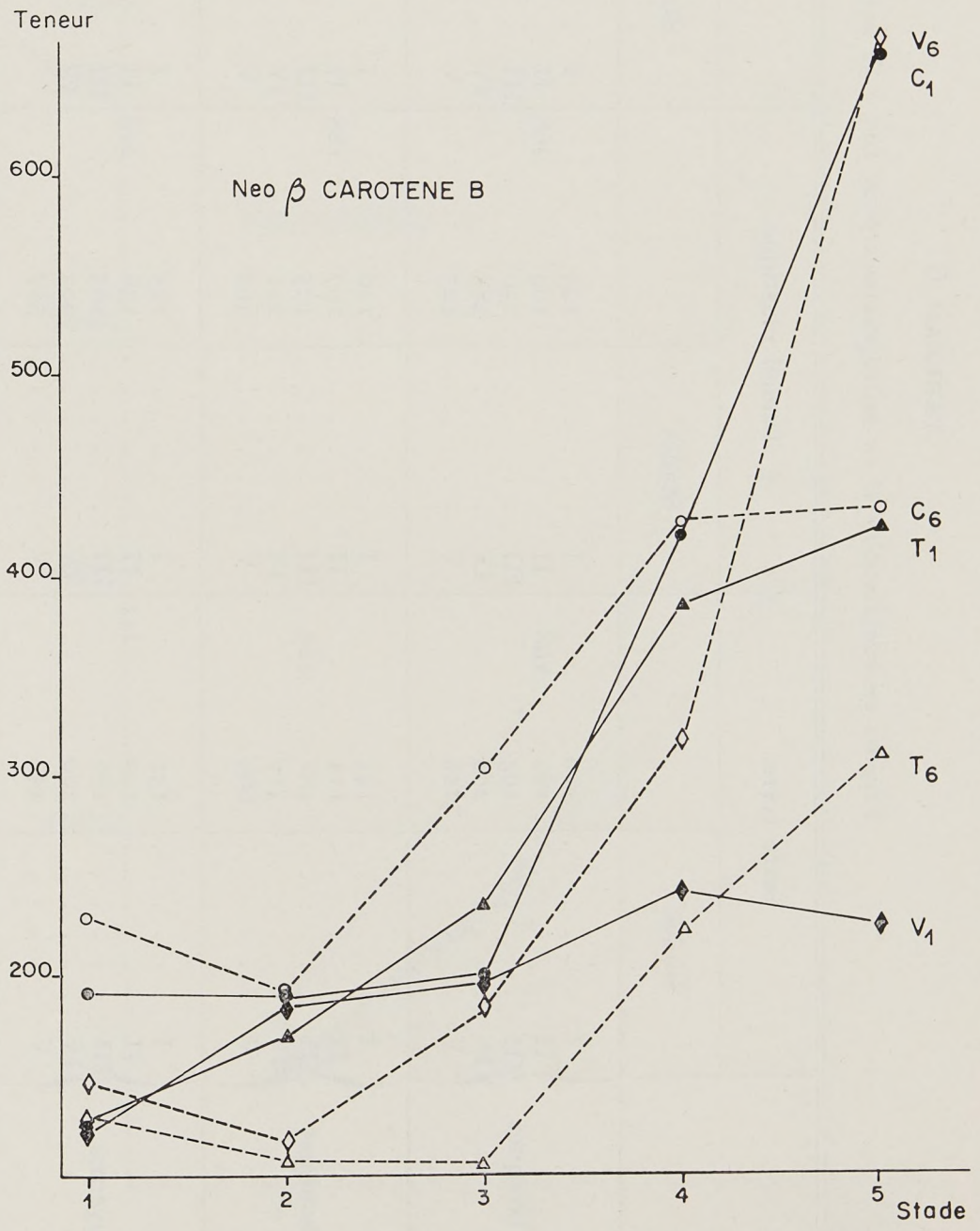

FIG. 4. — *Teneurs en néoβcarotène B en fonction de la maturation* (exprimées en microgrammes pour 100 g de matière sèche).

TABLEAU 6

Teneurs en néoβcarotène B en microgrammes pour 100 g de matière sèche

		Serre verre		Tunnel plastique		Plein champ		Moyenne	
	Stades			Stades		Stades			
1er bouquet	I	120		I 125		I 190		195	
	II	184	198	II 169	268	II 185	331	180	
	III	194		III 236		III 197		209	266
	IV	264		IV 385		IV 422		357	
	V	226		V 425		V 662		438	
6e bouquet	I	144		I 126		I 226		165	
	II	114		II 107	188	II 200	310	140	
	III	182	285	III 175		III 269		209	261
	IV	317		IV 221		IV 423		320	
	V	669		V 309		V 431		470	
Moyenne	I	132		I 125		I 208		155	
	II	149	241	II 138	228	II 192	321	160	
	III	188		III 205		III 233		209	
	IV	290		IV 303		IV 423		339	
	V	447		V 367		 547		454	

Position du bouquet

Les teneurs sont plus élevées pour le premier bouquet que le deuxième sous tunnel plastique, elles sont égales pour le premier et le sixième bouquet du plein champ. Les teneurs sont plus élevées sur le sixième bouquet que sur le premier en serre verre.

Stade de maturité

L'augmentation des teneurs se produit dans tous les modes de culture à partir du troisième stade, les deux premiers stades étant peu différents l'un de l'autre.

3.5. — *Néolycopène A* (tabl. 7, fig. 5)

Mode de culture

Les teneurs sont plus élevées sous verre serre qu'en tunnel plastique et plein champ.

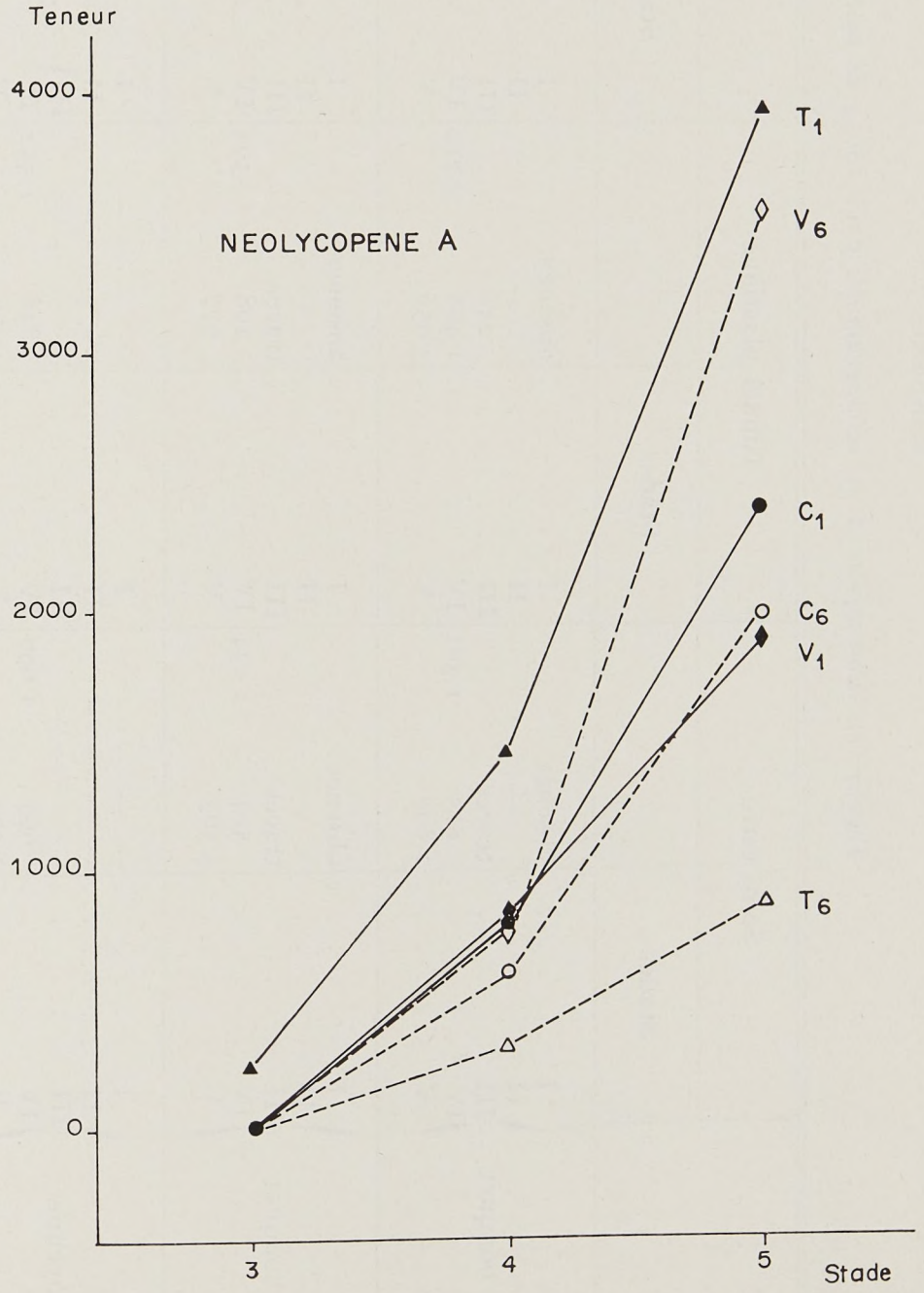

FIG. 5. — *Teneurs en néolycopène A en fonction de la maturation* (exprimées en microgrammes pour 100 g de matière sèche).

TABLEAU 7

Teneurs en néolycopène A en microgrammes pour 100 g de matière sèche

		Serre verre		Tunnel plastique		Plein champ		Moyenne	
	Stades			Stades		Stades			
1er bouquet	I	absence		I	absence	I	absence	— — — —	
	II	—		II	—	II	—	— — — —	
	III	traces		III	248	III	traces	— — — —	
	IV	825	1 361	IV	1 468	2 712	IV	790	1 601	1 027	
	V	1 898		V	3 956		V	2 416		2 756	1 892
6e bouquet	I	absence		I	absence	I	absence	— — — —	
	II	—		II	—	II	—	— — — —	
	III	traces		III	traces	III	traces	— — — —	
	IV	504	2 034	IV	208	392	IV	602	1 296	438	
	V	3 563		V	577		V	1 990		2 043	1 241
Moyenne	I	— — — —		I	— — — —	I	— — — —		
	II	— — — —		II	— — — —	II	— — — —		
	III	— — — —		III	— — — —	IIII	— — — —		
	IV	664	1 697	IV	838	1 552	IV	696	1 449	733	
	V	2 730		V	2 266		V	2 201		2 399	

TABLEAU 8

Teneurs en lycopène en microgrammes pour 100 g de matière sèche

		Serre verre		Tunnel plastique		Plein champ		Moyenne	
	Stades			Stades		Stades			
1er bouquet	I	absence		absence		absence		— — — —	
	II	—		—		—		— — — —	
	III	traces		470		traces		— — — —	
	IV	3 442	5 069	6 911	8 330	4 713	7 684	5 022	
	V	6 697		9 749		10 655		9 034	7 028
6e bouquet	I	absence		absence		absence		— — — —	
	II	—		—		—		— — — —	
	III	traces		traces		traces		— — — —	
	IV	3 745	3 654	1 394	2 195	2 846	7 881	2 661	
	V	3 695		4 196		12 915		6 891	4 776
Moyenne	I	— — — —		— — — —		— — — —			
	II	— — — —		— — — —		— — — —			
	III	— — — —		— — — —		— — — —			
	IV	3 593	4 362	4 152	5 562	3 779	7 782	3 842	
	V	5 130		6 972		11 785		7 963	

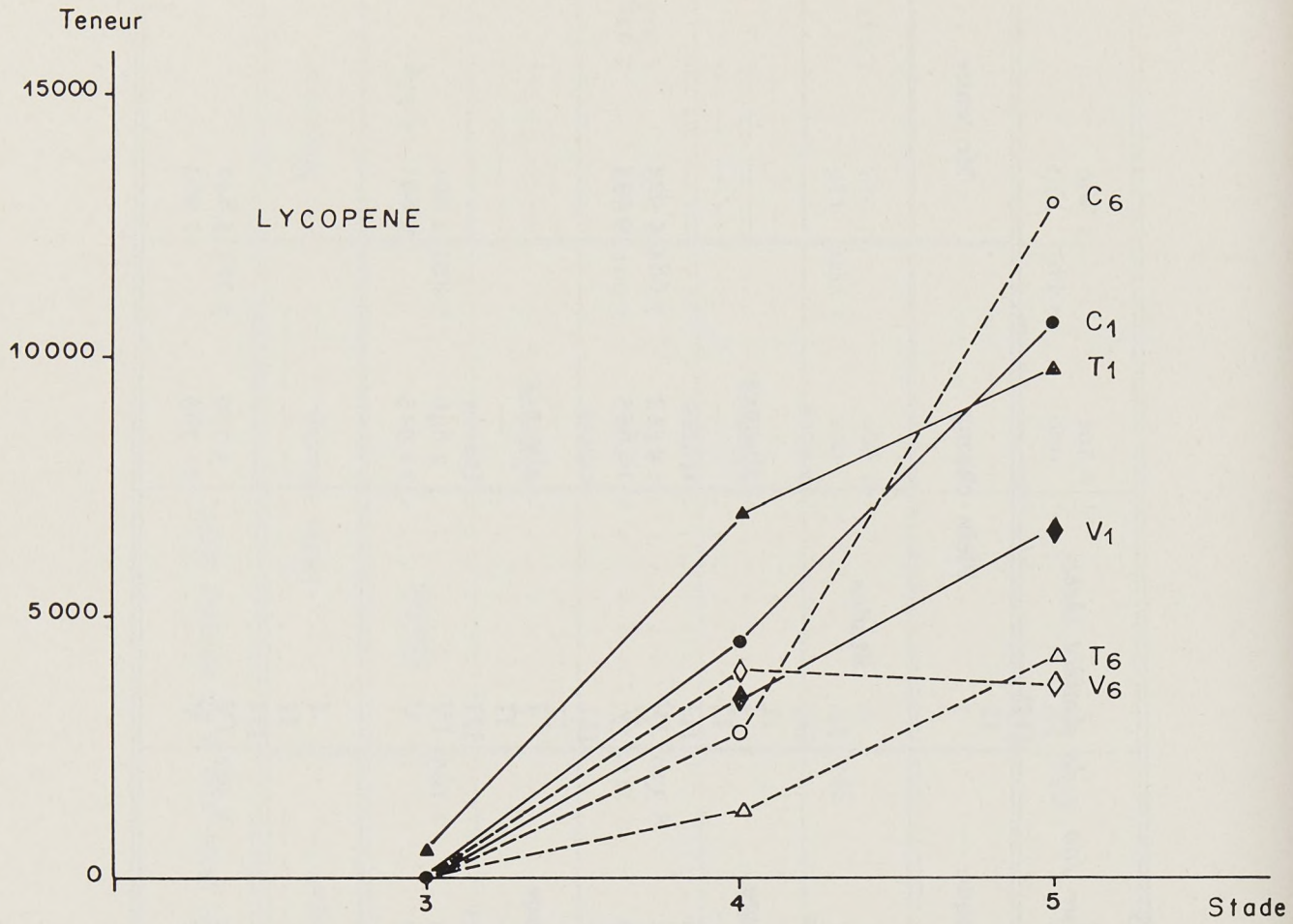

FIG. 6. — *Teneurs en lycopène en fonction de la maturation* (exprimées en microgrammes pour 100 g de matière sèche).

Position du bouquet

Les teneurs en néolycopène sont supérieures pour le sixième bouquet de la serre en verre par rapport au premier bouquet. On a l'inverse pour le tunnel plastique et le plein champ avec des teneurs nettement anormales pour le sixième bouquet du tunnel plastique.

Stades de maturité

Les teneurs au stade 5 sont environ 3 ou 4 fois plus élevées qu'au stade 4 dans tous les cas.

3.6. — *Lycopène* (tabl. 8, fig. 6)

Mode de culture

La teneur la plus faible se trouve dans les cultures faites sous serre verre, la plus forte est en plein champ, sous tunnel plastique, la teneur est intermédiaire mais plus proche de la serre verre.

Position du bouquet

Les teneurs en lycopène sont inférieures pour le sixième bouquet par rapport au premier dans les cultures sous abris. Elles sont égales pour le premier et le sixième bouquet en plein champ.

Stades de maturité

Le stade 5 est toujours supérieur au stade 4 mais dans des proportions beaucoup plus considérables pour les cultures en plein champ. Toutes les interactions, mode de culture-position du bouquet, mode de culture-stade de maturité, position du bouquet-stade de maturité sont significatives à 5 p. 100, sauf l'interaction position du bouquet-stade de maturité de la luteine.

IV. — Discussion et conclusion

L'étiquetage des fleurs avait pour but de constituer un échantillon de 90 fruits de même âge au même stade. Toutefois, le nombre limité de plants dont nous disposions n'a pas permis de réaliser cet objectif. En effet, d'un fruit à l'autre, le délai qui sépare la fécondation, du stade de maturité choisi présente des variations pouvant atteindre quinze jours, même pour un même bouquet.

Les conditions culturales dans lesquelles nous nous sommes placés ont été différentes d'un essai à l'autre quant au :

— Lieu de production donc nature du terrain;
— Époque d'évolution;
— Durée d'évolution;
— Conditions d'environnement (température, éclairement).

Cependant nous avons constaté que :

En valeur absolue, les teneurs en caroténoïdes sont en général fonction des conditions culturales; les tomates de plein champ présentent des teneurs en caroténoïdes supérieures à celles des tomates ayant poussé sous serre plastique ou verre.

Les caractères de l'environnement climatique tels que température ou rayonnement global ont une action prépondérante sur la biosynthèse de même que la nature du sol. En effet, pour les fruits provenant de la serre en verre et du plein champ, les teneurs en caroténoïdes des fruits du premier bouquet sont inférieures ou égales à celles du sixième bouquet, c'est l'inverse qui se produit pour les fruits du tunnel plastique. Ceci peut être expliqué par le fait que le sixième bouquet, du tunnel plastique a supporté une période de chaleur prolongée au mois de juin (« sécheresse » de 1976) on constate que les teneurs en caroténoïdes des fruits du sixième bouquet du tunnel plastique sont inférieures à celles du sixième bouquet de la serre verre, alors que pour le premier ces teneurs sont inférieures sous serre verre. Cette explication est d'autant plus vraisemblable que l'on constate des anomalies de la synthèse du lycopène, carotène dont la synthèse ne se fait plus au dessus de 30°. Les sixième bouquets des cultures sous abri montrent une chute importante de la teneur en lycopène par rapport au premier bouquet or, le mois de juin 1976 a eu 15 jours de températures supérieures à 30°, allant même jusqu'à 33°, qui ont dû toucher particulièrement les cultures sous abri.

La cinétique d'évolution est indépendante des modes de culture et de la place du fruit sur la plante.

Tous les caroténoïdes jaunes présentent une période de stabilité ou même dans certains cas de diminution entre les stades vert et jaune. Le lycopène aug-

mente très fortement entre le stade rose et rouge. Ces faits permettent d'envisager l'utilisation des caroténoïdes comme critère de stade physiologique de la tomate de façon à déterminer un stade optimal de récolte et de traitement en vue de la conservation.

En ce qui concerne les voies de biosynthèse de ces pigments, le fait que le βcarotène apparaisse avant le lycopène au cours de la maturation exclut la voie lycopène-βcarotène dans les premiers stades. Il ne peut provenir que de la cyclisation du neurosporène, le précurseur direct du lycopène.

Nous pouvons formuler deux hypothèses pour expliquer l'apparition tardive du lycopène :

1) L'équipement enzymatique permettant la transformation neurosporène-lycopène n'existe pas aux premiers stades de maturité.

2) Cet équipement existe mais il y a inhibition au profit de la voie neurosporène-carotènes cycliques par un produit naturel, manque d'un cofacteur ou retroinhibition par le β carotène.

Il sera donc intéressant d'étudier ultérieurement les voies de synthèse du lycopène et du β carotène à partir du neurosporène.

Reçu pour publication en juin 1980.

Summary

Evolution of carotenoids in tomatoes as a function of ripeness levels and cultural conditions

The effects of three cultural methods : glass and plastic greenhouse, field, as also the place of the fruit on the plant, on the evolution of carotenoïds amount of the tomato variety H63-5 were investigated.

Lycopene and βcarotene are suitable to be used as test for differentiating physiological steps of tomato ripeness. Indeed, the factors taken in account have no effect on the evolution of these two carotenoïds. They are only different in amount.

Remerciements

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Évaluation d'une méthode rapide d'assimilation des sucres (API 50 CH) en vue de l'identification des levures

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Résumé

Une nouvelle méthode d'étude de l'assimilation des sucres (API 50 CH) a été utilisée pour l'identification de cent cinquante et une souches de levures isolées du fromage de Camembert. Cette méthode peut se substituer valablement aux techniques classiques et elle représente un gain de temps important. Cependant, pour une identification correcte des espèces de levures, elle doit être employée conjointement avec les techniques usuelles d'observation microscopique et les épreuves de fermentation des sucres, de sporulation et de filamentation.

Mots clés : *levures — identification — assimilation sucres — nouvelle méthode —*

Introduction

L'identification des levures représente un problème qui, la plupart du temps, ne peut être résolu de façon simple ou rapide. En effet, sous le nom de levures, on regroupe actuellement quatre cent trente neuf espèces appartenant à cinquante sept genres différents (BARNETT *et al.*, 1979).

Dans les laboratoires de recherches, l'identification de ces microorganismes peut être réalisée selon les méthodes préconisées par deux « écoles » :

— « l'école anglaise », où l'on tient compte essentiellement des aptitudes biochimiques (fermentation et assimilation des sucres ou d'autres substrats) et de quelques caractères physiologiques (croissance à 37 °C, croissance en l'absence de vitamines...). L'identification, dans ce cas, se réalise selon la clé de BARNETT et PANKHURST (1974) qui a été récemment complétée par certains tests morphologiques et culturaux (BARNETT *et al.*, 1979).

— « l'école hollandaise », la plus ancienne, où l'on enregistre l'aspect des cultures, tant en milieu liquide que solide, la forme et la taille des cellules, l'aptitude ou non à la formation de filaments mycéliens, l'aptitude à la formation d'ascospores ainsi que la forme et le nombre des spores, la fermentation et l'assimilation des sucres et de divers composés. L'identification est alors conduite selon la clé proposée par LODDER (1971).

Cette méthode est sûre, mais sa mise en œuvre est longue, les lectures doivent parfois s'étendre sur plusieurs semaines et, en pratique courante, elle est difficilement utilisable.

Un certain nombre de clés dites simplifiées ont été proposées (BEECH *et al.*, 1968) mais elles restent encore relativement lourdes dans leur application. Aussi, se plaçant d'un point de vue pratique, une firme commerciale française a-t-elle essayé de mettre au point un type de galerie rapidement exploitable pour les réponses aux tests d'assimilation des sucres et d'autres composés.

Le système que nous avons présentement employé est le système API 50 CH (50 carbohydrates) (*). Il a été éprouvé sur cent cinquante et une souches de levures isolées à partir de fromages à pâte molle de type Camembert (SCHMIDT et LENOIR, 1980) identifiées selon les critères classiques de LODDER (1971) et de BARNETT et PANKHURST (1974).

Protocole expérimental

1. — *Description du dispositif*

Le système API 50 CH est une microméthode standardisée de recherche du pouvoir assimilatif, applicable à divers microorganismes (GRILLOT, 1974).

Le dispositif se présente sous forme d'une galerie de cinquante micro-cuves ou cupules, dérivant du tube de Ivan-Hall (BUISSIÈRE et NARDON, 1968; BUISSIÈRE, 1972), dont le fond est constitué d'un support contenant le substrat approprié sous forme déshydratée.

L'assimilation est décelée par l'observation de la turbidité.

La miniaturisation et la simplicité du dispositif permettent d'effectuer de nombreux tests en un temps court, et il est ainsi possible d'étudier un nombre important de souches.

Aucune précaution d'aseptie n'est nécessaire car le temps d'incubation est limité et la charge en cellules microbiennes, inoculées par cupule, est élevée.

La galerie utilisée permet la recherche du pouvoir assimilatif sur quarante neuf carbohydrates (tabl. 1).

2. — *Mode opératoire*

Les souches de levures étudiées (tabl. 2) sont cultivées 72 heures à 25 °C sur milieu Y.E.G. (extrait de levure, 0,5 p. 100; glucose, 2 p. 100; gélose, 2,5 p. 100). Une suspension de la souche est alors réalisée en solution de chlorure de sodium à

(*) API System : La Balme les Grottes, 38390 Montalieu Vercieu (France).

TABLEAU I

Carbohydrates de la galerie API 50 CH

0 Témoin	25 Esculine
1 Glycérol (*)	26 Salicine (*)
2 Erythritol (*)	27 D Cellobiose (*)
3 D Arabinose (*)	28 Maltose (*)
4 L Arabinose (*)	29 Lactose (*)
5 Ribose (*)	30 D Melibiose (*)
6 D Xylose (*)	31 Saccharose (*)
7 L Xylose	32 Trehalose (*)
8 Adonitol	33 Inuline (*)
9 Méthyl Xyloside	34 D Melezitose (*)
10 Galactose (*)	35 D Raffinose (*)
11 D Glucose	36 Amidon (*)
12 D Fructose	37 Glycogène
13 D Mannose	38 Xylitol
14 L Sorbose (*)	39 β Gentiobose
15 Rhamnose (*)	40 D Turanose
16 Dulcitol	41 D Lyxose
17 Inositol (*)	42 D Tagatose
18 Mannitol (*)	43 D Fucose
19 Sorbitol	44 L Fucose
20 Méthyl D Mannoside	45 D Arabitol
21 Méthyl D Glucoside (*)	46 L Arabitol
22 N Acétyl Glucosamine	47 Gluconate
23 Amygdaline	48 2 Ceto Gluconate (*)
24 Arbutine (*)	49 5 Ceto Gluconate (*)

(*) Carbohydrates inclus dans la grille d'identification de BARNETT et PANKHURST (1974).

TABLEAU 2

Liste des souches éprouvées avec le système API 50 CH

Espèces	Nombre de souches
Saccharomyces italicus	64
Debaryomyces hansenii	31
Kluyveromyces lactis	8
Torulopsis sphaerica	24
Kluyveromyces fragilis	6
Candida pseudotropicalis	7
Kluyveromyces bulgaricus	11

9 p. 1 000 de façon à obtenir une densité optique correspondant à l'échelle VI de Mac Farland, et mélangée, volume à volume, avec du Bacto Yeast Nitrogen Base Difco nº 392 gélosé à 14 p. 1 000 et amené vers 45 °C. Quelques gouttes de cette préparation sont alors introduites, à la pipette Pasteur, dans chacune des cupules de la galerie. Avant mise à l'étuve à 30 °C, les galeries sont placées dans des boîtes d'incubation contenant environ 5 ml d'eau pour empêcher la déshydratation. Les lectures sont réalisées après 48 et 72 heures d'incubation. Les assimilations sont notées positives lorsque la turbidité est supérieure à celle du témoin.

Préparation de l'échelle optique de Mac Farland

L'échelle de Mac Farland est préparée en introduisant différentes quantités de chlorure de baryum à 1 p. 100 et d'acide sulfurique à 1 p. 100 dans dix tubes à essais de diamètre uniforme. Le tube 1 reçoit 0,1 ml de la solution de chlorure de baryum, le tube 2, 0,2 ml..., le tube 10, 1 ml. Le volume, dans chaque tube, est amené à 10 ml par addition d'acide sulfurique. Les tubes, avant emploi, sont agités, et la suspension microbienne examinée est placée dans un tube de même calibre pour effectuer la comparaison.

Résultats et discussion

L'étude du pouvoir assimilatif des cent cinquante et une souches de levures éprouvées montre que la concordance entre les réponses obtenues par l'utilisation des galeries API 50 CH et les tests classiques d'assimilation en milieu liquide est, après 48 heures d'incubation à 30 °C, de 92 p. 100 et, après 72 heures d'incubation, de 96 p. 100 pour les hydrates de carbone mentionnés dans la grille d'identification de Barnett et Pankhurst (1974) (tabl. 3). Des incubations plus longues, de l'ordre d'une semaine, n'apportent pas de réponses supplémentaires et elles sont à l'origine du développement de germes de contamination.

Concernant ces pourcentages de réponses concordantes, il convient de noter deux exceptions : le raffinose, avec des concordances qui atteignent seulement 70 p. 100 après 48 heures et 82 p. 100 après 72 heures, et le tréhalose, respectivement 67 et 76 p. 100. Ces chiffres rejoignent ceux cités par Gilles et Guinet (1978) dans leur évaluation de la microméthode d'identification des levures API 20 C.

Le nombre de fois où l'identification a pu être convenablement conduite, sur les souches analysées, avec comme seule base les résultats obtenus sur la galerie API 50 CH, est restreint. On enregistre seulement 9 p. 100 d'identifications positives après 48 heures d'incubation, et 16 p. 100 après 72 heures. En revanche, sur des levures d'origine médicale, les résultats corrects d'identification paraissent être très supérieurs. Ainsi, dans une étude des genres *Candida, Torulopsis, Cryptococcus...*, Land *et al.* (1979), utilisant les nouvelles galeries API 20 C (dont le principe est analogue aux galeries 50 CH, mais qui ne renferment que 19 substrats), ont pu procéder à l'identification de 75 p. 100 des souches éprouvées. Par cette même méthode, De Louvois *et al.* (1979) ont enregistré 100 p. 100 d'efficacité pour l'identification de *Candida albicans* et 83 p. 100 pour celle d'autres espèces de *Candida.*

Indépendamment du pourcentage d'identifications correctes, deux limites paraissent liées au principe même et à l'utilisation de ce type de méthode.

Ainsi, il apparaît (tabl. 1) que l'on ne connaît la réponse théorique que pour vingt sept des tests prévus dans la galerie 50 CH. De plus, il importe de tenir compte de la validité des différentes épreuves biochimiques utilisées dans la classification (Barnett et Pankhurst, 1974) (tabl. 3) et si seize tests possèdent un bon coefficient de discrimination, il n'en est pas de même pour les autres. Ceci conduit, en se référant aux méthodes classiques, à regretter l'absence de certaines épreuves particulièrement utiles dans l'identification des levures, les assimilations du nitrate, du ribitol, du glucitol, du citrate et du succinate... par exemple.

TABLEAU 3

Comparaison des résultats des tests d'assimilation obtenus par la galerie API 50 CH et les méthodes classiques

Tests API 50 CH utilisables en classification (1)	Réponses correctes obtenues en % des réponses théoriques (2)		Validité du test (*) (3)
	48 h – 30 °C	72 h – 30 °C	
Glycérol	96	98	3,3
Érythritol	95	98	64
D-Arabinose	93	97	1,5
L-Arabinose	93	96	6,0
Ribose	94	97	0,49
D-Xylose	94	97	26
Galactose	96	99	18
D-Glucose	97	99	0
L-Sorbose	94	97	2,9
Rhamnose	96	98	4,0
Inositol	94	97	37
Mannitol	93	95	27
Méthyl-D-Glucoside	96	98	0,58
Arbutine	96	99	< 0,01
Salicine	95	98	1,6
D-Cellobiose	93	97	76
Maltose	96	99	100
Lactose	96	98	37
D-Melibiose	94	98	32
Saccharose	94	97	36
Tréhalose	67	76	5,2
Inuline	88	90	6,1
D-Melezitose	93	96	9,3
D-Raffinose	70	82	27
Amidon	92	97	3,0
2-Céto-Gluconate	92	95	0,99
5-Céto-Gluconate	93	97	0
Moyenne	92 %	96 %	

(*) Validité du test calculée en prenant l'assimilation du maltose comme référence (base = 100).
(1), (3) : Selon BARNETT et PANKHURST (1974), p. 19 et 20.
(2) : Moyenne des réponses des souches des différentes espèces de levures éprouvées.

Toutefois, il n'en reste pas moins qu'en combinant la galerie API 50 CH avec les méthodes classiques d'étude pour la morphologie, les aspects culturaux, la mise en évidence de l'aptitude à la filamentation et à la sporulation, la fermentation des sucres, on relève un gain de temps important et des facilités de manipulation certaines, surtout lorsque l'identification porte sur un grand nombre de souches. Dans ce cas, ce type de galerie semble pouvoir être substitué avec intérêt aux méthodes classiques d'assimilation des sucres (méthode auxanographique ou technique en milieu liquide) qui sont relativement lourdes à mettre en œuvre et dont les réponses ne peuvent, en général, être enregistrées qu'après un temps assez long (8 jours à 3-4 semaines).

Une autre voie d'utilisation de cette galerie paraît possible. On peut, par exemple, envisager un couplage des réponses obtenues sur galerie API 50 CH avec celles fournies par les systèmes API 50 AA (50 acides aminés) et API 50 AO (50 acides organiques). Il est permis de penser qu'en appliquant alors les méthodes de taxonomie numérique, c'est-à-dire en traitant les informations collectées par ordinateur et en se référant aux réponses fournies par un certain nombre de souches-types, l'identification des levures pourrait être conduite de façon correcte plus rapidement.

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Remerciements

Nous remercions vivement la Société API SYSTEM pour la mise à notre disposition du matériel d'étude.

Summary

Evaluation of a new API 50 CH strip for yeast identification by sugar assimilation

A new method for the study of sugar assimilation (API 50 CH) was proved for the identification of 151 yeast strains isolated from Camembert cheese. This method can be used instead of standard technics and it saves a great deal of time.

Nevertheless it has not to be employed alone but together with usual microscopic observation, sugar fermentation, sporulation and forming filaments tests for accurate identification of yeasts.

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Caractéristiques des amidons de plantes alimentaires tropicales : Action de l'alpha-amylase, gonflement et solubilité

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Résumé

Trente amidons de plantes alimentaires tropicales de divers genres espèces et variétés sont étudiés du point de vue de leur sensibilité à l'hydrolyse alpha-amylasique et de leurs propriétés de gonflement et de solubilité dans l'eau en fonction de la température. Ces différentes caractéristiques apparaissent très variables en fonction du genre botanique, mais aussi de l'espèce et parfois des cultivars.

Leurs relations éventuelles entre elles et avec la taille des grains d'amidons, la teneur en amylose et le type d'organisation cristalline sont discutées en regard des résultats acquis chez les amidons de maïs et de pommes de terre.

En général, il semble que les amidons à petits grains et à faible teneur en amylose sont plus sensibles à l'action de l'alpha-amylase que les autres amidons et possèdent les vitesses de solubilisation les plus faibles par rapport aux vitesses de gonflement.

Mots clés : *amidons tropicaux, alpha-amylolyse, gonflement, solubilité dans l'eau.*

1. — Introduction

Dans une précédente publication (DELPEUCH *et al.*, 1978), la composition chimique, la morphologie et les spectres de diffraction de rayons X de plusieurs amidons tropicaux ont été décrits et discutés. Le présent article traite de leur comportement au cours d'une alpha-amylolyse et de leurs propriétés de gonflement et solubilité dans l'eau en fonction de la température. Les relations entre ces propriétés et les caractéristiques déjà étudiées font l'objet d'une attention particulière.

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2. — Matériel et méthodes

Sur les amidons de maïs, pomme de terre et manioc extraits au laboratoire, pris en référence, et sur les amidons de plantes tropicales purifiés précédemment décrits (DELPEUCH *et al.*, 1978) ont été étudiés :

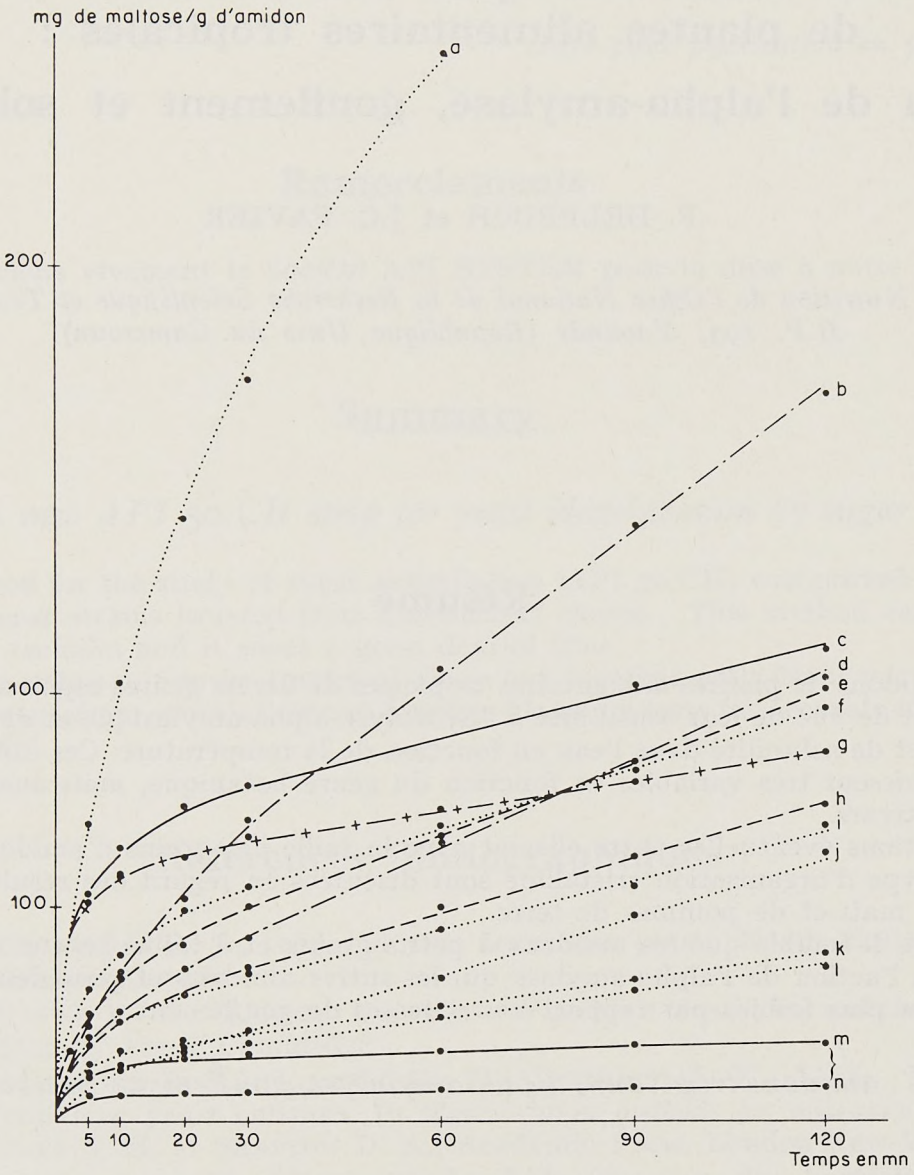

FIG. 1. — *Cinétiques de la dégradation α-amylasique d'amidons crus tropicaux*

— . — . — . —	témoins	*Zea mays*	b
		Manihot	d
— — — — — —	*D. dumetorum*	*D. dumetorum* blanc 1971	e
		D. dumetorum hirsute jaune 1970	h
— + — + — +	*D. esculenta*		g
———	amidons à spectre B	*Artocarpus communis*	c
		D. rotundata ogoja	m
		D. cayenensis digité	n
		Plupart des *dioscorea*	m à n
.....	amidons à spectre A et C a	*Pachyrrhizus*	a
		Colocasia blanc	f
		Colocasia rouge	i
		Ipomea jaune	j
		Artocarpus integra	k
		Coleus	l
		Xanthosoma (confondu à *D. rotundata* ogoja)	m

— La sensibilité des amidons à une alpha-amylase d'origine bactérienne, déterminée par la méthode de Tollier et Guilbot (1971) : une suspension à 2,5 p. 100 d'amidon sec est additionnée de 20 p. 100 (proportion rapportée à la masse pondérale d'amidon sec) d'alpha-amylase (de la firme N.B.C.) en tampon phosphate 0,005 M; les glucides alcoolo-solubles libérés sont ensuite dosés par une méthode à l'anthrone (Lœwus, 1952) et exprimés en mg de maltose par g d'amidon initial.

— Les gonflements et solubilités des amidons dans l'eau, en fonction de la température, déterminés de 5 en 5 °C par la méthode de Leach *et al.* (1959) modifiée par Mercier, 1968.

3. — Résultats

3.1. — *Sensibilité des amidons à l'alpha-amylase bactérienne*

Les cinétiques d'amylolyse des amidons crus sont représentés sur les figures 1 et 2.

TABLEAU I

Caractéristiques des courbes d'alpha-amylolyse des amidons d'ignames

Origine de l'amidon	Vitesse initiale Vi	Vitesse finale Vf	Fraction facilement hydrolysable FH
Ignames			
D. rotundata Ogoja	1,12	0,02	1,4
D. rotundata conique	0,68	0,60	0,7
D. cayenensis long	0,69	0,01	0,8
D. cayenensis digité	0,52	0,01	0,6
D. schimperiana	0,91	0,02	1,0
D. libreschiana	0,63	0,02	0,7
D. bulbifera ex Gwofon	0,90	0,03	1,0
D. alata ex Bafut	0,65	0,02	0,7
D. esculenta	5,10	0,12	5,9
D. dumetorum hirsute jaune (Bambui 70)	1,36	0,18	1,4
D. dumetorum hirsute jaune (Yaoundé 71)	1,76	0,20	2,5
D. dumetorum lisse jaune (Bambui 70)	1,92	0,23	2,2
D. dumetorum lisse jaune (Bambui 71)	2,06	0,25	2,6
D. dumetorum lisse blanc (Bambui 70)	2,15	0,29	2,6
D. dumetorum lisse blanc (Yaoundé 71)	2,45	0,30	2,9

Vi = amidon dégradé pendant les 5 premières minutes, p. 100 d'amidon initial.
Vf = amidon dégradé en moyenne en 5 minutes pendant la première heure, p. 100 d'amidon initial.
FH = extrapolation de la partie linéaire des courbes.

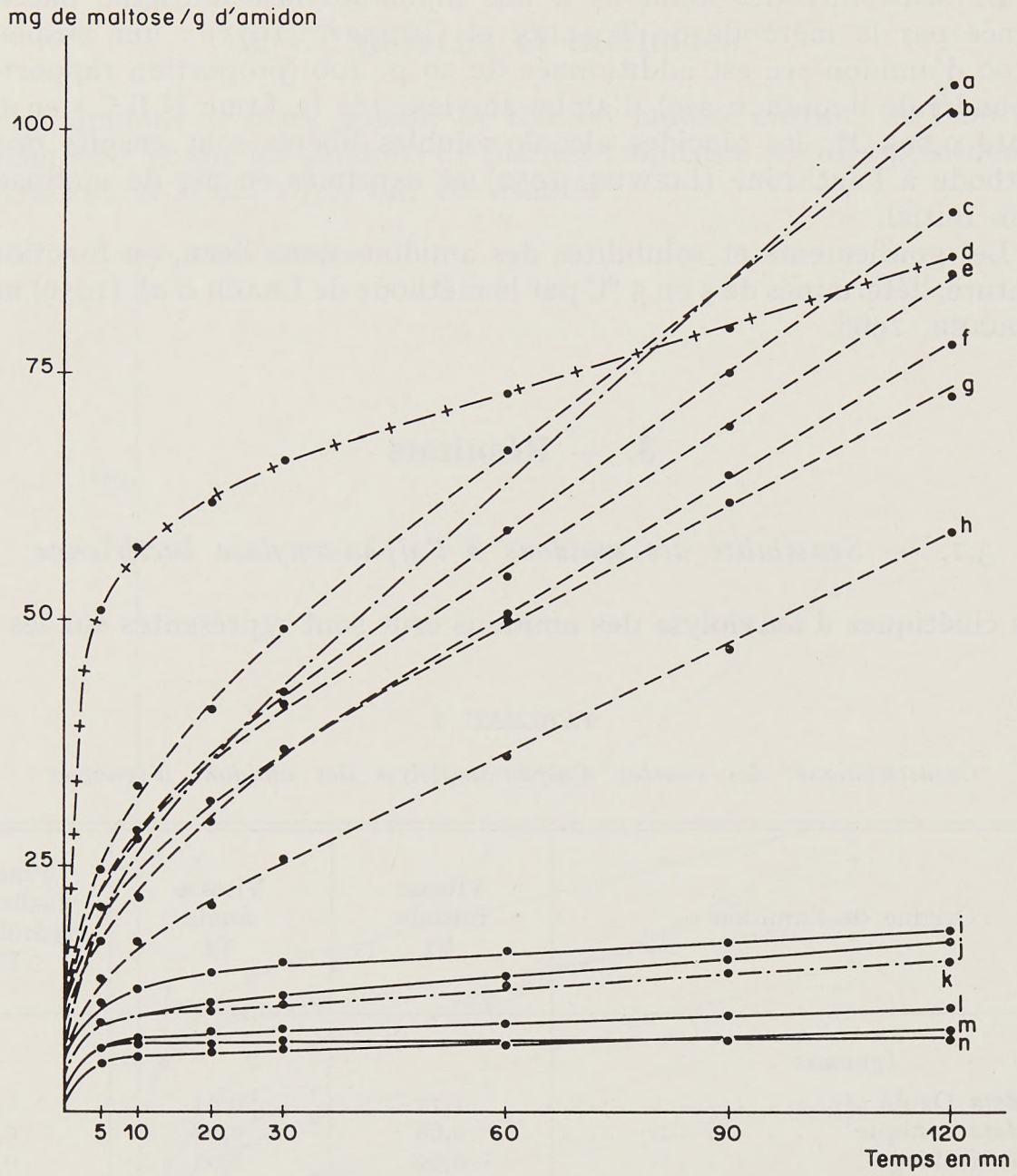

FIG. 2. — *Cinétiques de la dégradation α-amylasique d'amidons crus d'ignames comparées à celles des amidons de manioc et de pomme de terre*

D. dumetorum D. dumetorum Pachyrrhizus

— . — . — . —	témoins *Manihot*	a
	Solanum	k
———————	*D. dumetorum* lisse blanc 1971	g
	lisse blanc 1970	c
	lisse jaune 1971	e
	lisse jaune 1970	f
	hirsute jaune	g
	hirsute jaune	h
— + — + — +	*D. esculenta*	d
———————	amidons à spectre B *D. rotundata* agoja	i
	D. bulbifera	j
	D. schimperiana (confondu à solanum)	k
	D. libreschiana	l
	D. cayenensis long	l
	D. alata	l
	D. cayenensis digité	m
	D. rotundata conique	n

TABLEAU 2

Caractéristiques des courbes d'alpha-amylolyse de divers amidons tropicaux

Origine de l'amidon	Vi	Vf	FH
Manioc			
Manihot esculenta			
rouge	1,58	0,30	1,6
blanc	1,90	0,35	2,2
Patates douces			
Ipomea batatas			
jaune ex-America	0,98	0,22	0,7
blanche ex-Menjo	1,00	0,30	0,7
rouge ex-Nkolbisson	0,93	0,24	0,7
Macabos			
Xanthosoma sagittifolium			
rouge	0,84	0,04	0,9
blanc	0,96	0,05	1,1
Taros			
Colocasia antiquorum			
blanc gros tubercules	3,15	0,22	4,2
rouge ex Nkambé	1,74	0,18	2,4
petite taille ex Bafut	3,16	0,16	4,9
Carotte locale			
Coleus esculentus	1,06	0,11	1,2
Haricot igname			
Pachyrrhizus erosus	6,90	1,20	10,2
Arbre à pain			
Artocarpus communis			
spyrena (pulpe)	4,90	0,13	5,5
seminifera (graines)	5,20	0,18	6,7
Jacquier			
Artocarpus integra (graines)	1,19	0,10	1,4

Pour comparer les différents tracés, nous avons déterminé les paramètres suivants (MERCIER, 1968) : vitesse initiale (Vi) et finale (Vf) d'hydrolyse, fraction facilement hydrolysable (FH). Les résultats sont rapportés aux tableaux 1 et 2.

Parmi les ignames (*Dioscorea*), les amidons des espèces *D rotundata, D. cayenensis, D. schimperiana, D. libreschiana, D. bulbifera et D. alata,* tous à spectre type B, sont très difficilement hydrolysables par l'alpha-amylase bactérienne, comme l'amidon de pomme de terre pris en référence et également à spectre type B. Dans tous les cas, les valeurs des paramètres Vi, Vf et FH sont comparables entre elles. Par conséquent, à l'état cru, tous ces amidons de tubercules apparaissent nettement plus difficiles à hydrolyser que les amidons crus de céréales.

Par contre, les amidons de l'espèce *D. dumetorum* apparaissent beaucoup plus facilement hydrolysables que ceux des autres espèces de *Dioscorea* : leurs courbes d'hydrolyse s'inscrivent entre celles des amidons de maïs et de pomme de

terre, comme la courbe relative à l'amidon de manioc; les vitesses initales et finales sont nettement plus élevées que pour les amidons des autres ignames, leurs valeurs étant voisines ou un peu supérieures à celles relatives à l'amidon de manioc (tabl. I et II). Pour chacune des trois variétés de *D. dumetorum*, l'amidon des tubercules récoltés en 1971 se montre plus facilement hydrolysable que celui des tubercules récoltés en 1970, mais il pourrait s'agir d'un artefact : les amidons de 1971 ont séjourné dans leur eau de lavage plus longtemps que ceux de 1970, leur récupération ayant été effectuée par simple décantation et non par centrifugation, d'où la possibilité d'un début de fermentation entrainant une légère dégradation.

Les amidons de *D. esculenta*, des deux *Artocarpus communis* et des *Colocasia antiquorum* blanc et ex-Bafut ont des courbes d'hydrolyse d'un aspect plus particulier : les vitesses initiales et les fractions facilement hydrolysables sont plus élevées, respectivement au voisinage de 5,1 et 5,9, que celles relatives aux autres amidons, alors que les vitesses finales gardent des valeurs faibles, voisines de 0,15. Les courbes d'hydrolyse des amidons de *D. esculenta et de D. dumetorum*, se différencient nettement des courbes données par les autres ignames.

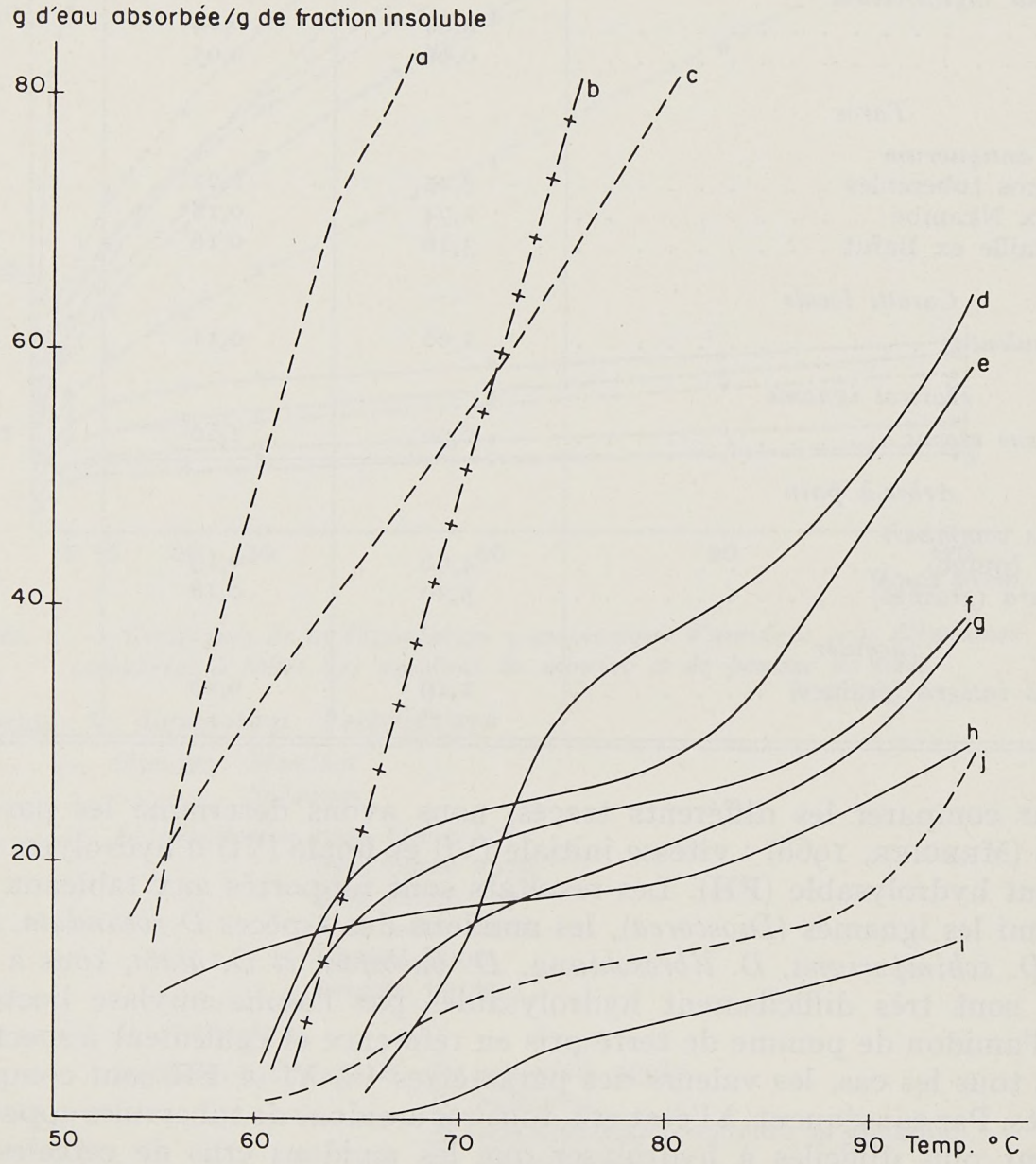

FIG. 3 — *Gonflement de divers amidons en fonction de la température*
a) *Solanum tuberosum*; b) D. *esculenta*; c) *Manihot esculenta*; d) *Dumetorum* hirsute jaune 1971; e) D. *rotundata* conique; f) D. *cayenensis* long; g) D. *alata*; h) D. *bulbifera*; i) D. *libreschiana*; j) *Zea mays*.

Après les amidons d'ignames, ce sont ceux de macabos et de patates douces qui sont les plus difficilement attaqués par l'enzyme. Les amidons de patates douces présentent cependant une vitesse finale d'amylolyse plus élevée. A cette catégorie d'amidons peu sensibles à l'action de l'alpha-amylase se rattache également l'amidon de *Coleus esculentus*.

L'amidon de *Pachyrrhizus erosus* présente une très grande sensibilité à l'hydrolyse; les paramètres Vi, Vf, FH sont de l'ordre de ceux observés pour un amidon de céréale tel que l'amidon de blé.

3.2. — *Gonflement et solubilité dans l'eau en fonction de la température*

L'évolution du gonflement en milieu aqueux des amidons de référence et des amidons étudiés est représentée sur les figures 3, 6, et 9 en fonction de la température; le gonflement, traduisant la capacité d'hydratation de la fraction d'amidon insoluble dans l'eau, est exprimé en grammes d'eau absorbée par gramme de fraction insoluble. L'évolution de la solubilité des amidons est représentée sur les fig. 3, 7 et 9 où la solubilité est exprimée en pourcentage d'amidon solubilisé.

Pour les amidons de référence, nos diagrammes sont comparables à ceux obtenus par plusieurs auteurs, (Leach *et al.* 1959, Hollo et Guilbot 1964, Mercier 1968) : le gonflement des amidons du manioc et de la pomme de terre commence à se produire vers 53 °C et 55 °C respectivement et s'accroît à peu près linéairement quand la température s'élève; celui de l'amidon de maïs présente

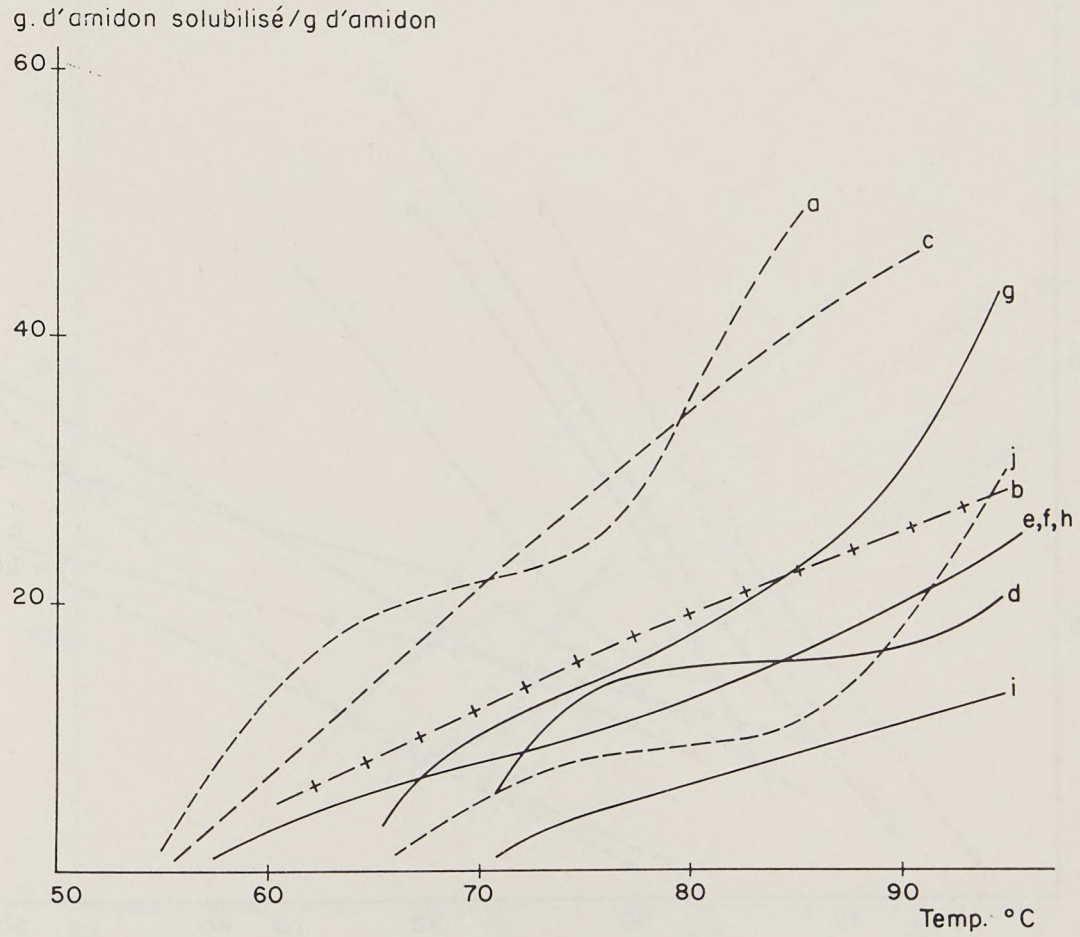

Fig. 4. — *Solubilité de divers amidons en fonction de la température*
a) *Solanum tuberosum*; b) D. *esculenta*; c) *Manihot esculenta*; d) *Dumetorum* hirsute jaune 1971; e) D. *rotundata* conique; f) D. *cayenensis* long; g) D. *alata*; h) D. *bulbifera*; i) D. *libreschiana*; j) *Zea mays*.

deux stades d'accroissement, le premier entre 60 et 65 °C et le second vers 90 °C. Par contre la capacité d'hydratation de l'amidon de maïs paraît assez limitée comparativement à celle des amidons de manioc et de pomme de terre. Cette différence se retrouve au niveau de l'importance de la solubilisation.

En ce qui concerne les ignames (fig. 3), l'amidon de *D. esculenta* se distingue de celui des autres espèces par son gonflement élevé et linéaire, comparable à celui du manioc et de la pomme de terre; le gonflement des amidons des autres espèces intervient en général à des températures supérieures, comprend deux stades et semble plus limité. Le gonflement des amidons des variétés de *D. dumetorum* subit un accroissement brusque vers 70 °C et paraît assez élevé sans toutefois se distinguer particulièrement de celui des autres espèces. L'amidon de *D. libreschiana*, à forte teneur en amylose, possède la capacité d'hydratation la plus faible.

Si l'on considère les diagrammes de solubilité des amidons d'ignames en fonction de la température (fig. 4), on observe qu'ils présentent une allure comparable à celle des diagrammes de gonflement. La solubilité est dans l'ensemble assez limitée et présente souvent deux stades comme dans le cas du maïs.

Les amidons des macabos, taros et patates douces (fig. 6 et 7) sont comparables à ceux des ignames du point de vue du gonflement et de la solubilité. L'amidon de taro petite taille possède cependant une capacité d'hydratation et une solubilité plus élevées.

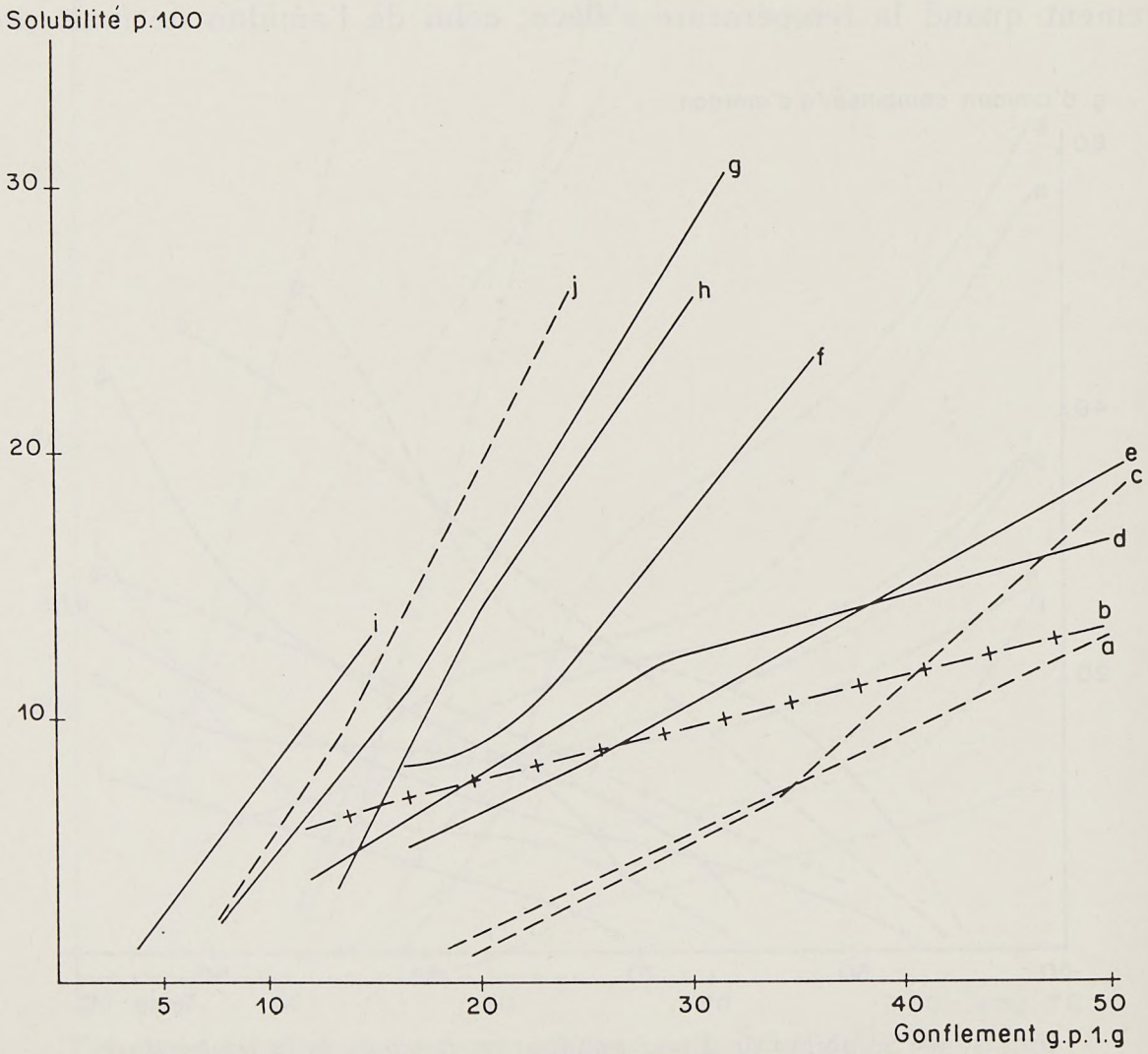

FIG. 5. — *Solubilité en fonction du gonflement de divers amidons*
a) *Solanum tuberosum*; b) D. *esculenta*; c) *Manihot esculenta*; d) *Dumetorum* hirsute jaune 1971; e) D. *rotundata* conique; f) D. *cayenensis* long; g) D. *alata*; h) D. *bulbifera*; j. *Zea mays*.

L'amidon de la pulpe du fruit d'*A. communis apyrena*, à faible teneur en amylose, se distingue des amidons des autres *artocarpus* — qui sont des amidons extraits des graines — par sa capacité d'hydratation importante et sa solubilité réduite (fig. 6 et 7).

Enfin, pour *Pachyrrhizus* et *coleus*, les gonflements et solubilités sont assez limités sans que l'on puisse, dans nos conditions expérimentales, distinguer deux stades d'accroissement (fig. 9).

Les graphiques de solubilité en fonction du gonflement (fig. 5) montrent que les amidons des ignames *D. esculenta* et *D. dumetorum*, comme l'amidon de pomme de terre, se solubilisent moins rapidement qu'ils ne gonflent. Pour un même stade de gonflement ces amidons, à faible teneur en amylose, présentent toujours une solubilité inférieure à celle des autres amidons d'ignames. Le même phénomène apparaît (fig. 8) pour l'amidon d'*A. communis apyrena* (9 p. 100 d'amylose) et, à un degré moindre, pour l'amidon du taro petite taille. Inversement, le gonflement de l'amidon de l'igname *D. libreschiana* (34,5 p. 100 d'amylose) comme celui des amylomaïs (Mercier, 1968) reste très réduit pour un taux de solubilisation assez important.

En définitive on peut dire que, généralement, la capacité d'hydratation et la

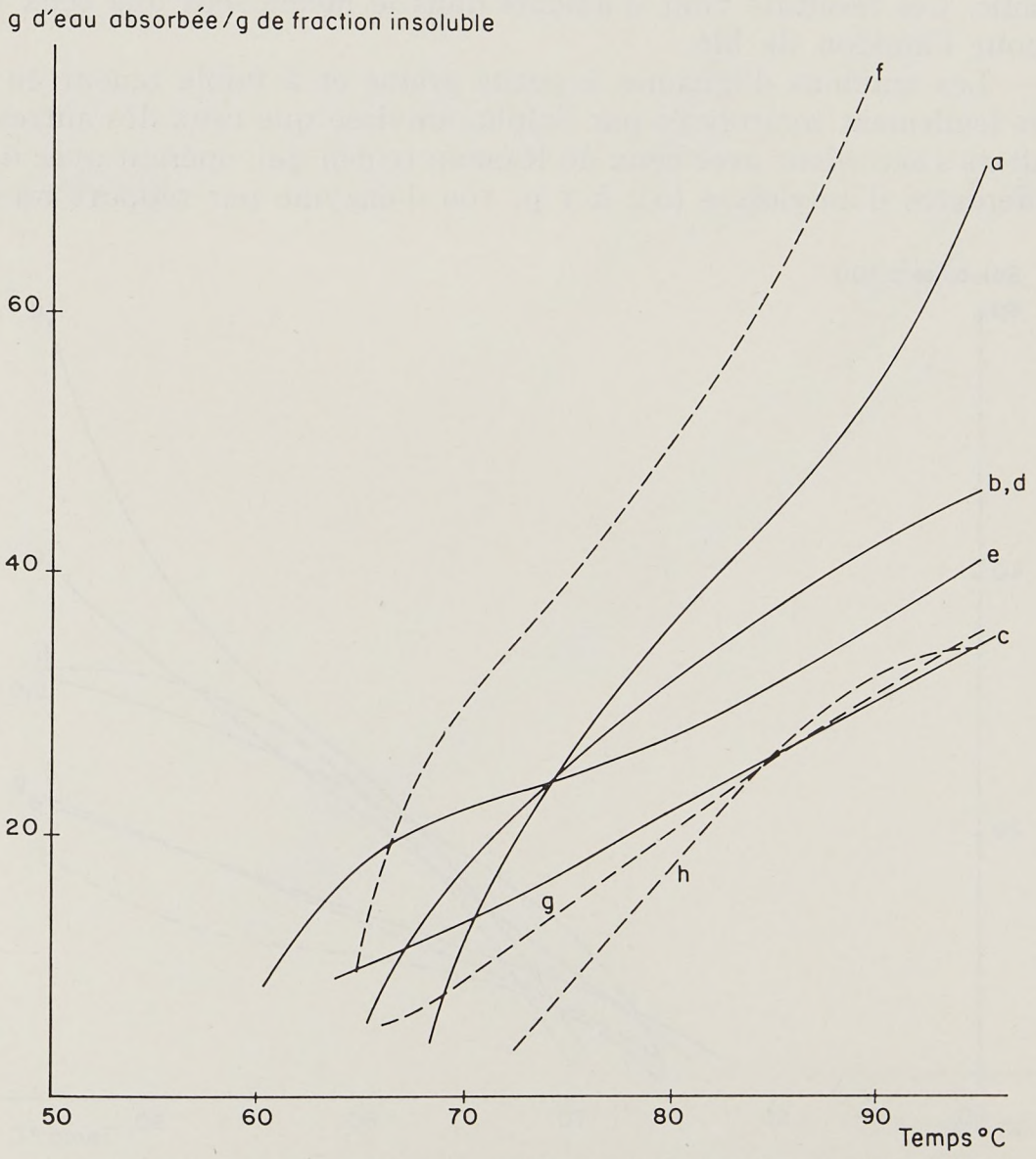

Fig. 6. — *Gonflement de divers amidons en fonction de la température*
a) *Colocasia* petite taille; b) *Colocasia* blanc; c) *Colocasia* rouge; d) *Xanthosoma* rouge; e) *Ipomea* jaune; f) *A. communis apyrena*; g) *A. communis seminefera*; h) *A. integra*.

solubilité de l'ensemble des amidons des plantes alimentaires tropicales étudiées ici sont comparables à celles du maïs, mais sont plus réduites et se manifestent à des températures plus élevées que celles des amidons de pomme de terre et de manioc.

4. — Discussion et conclusion

Les résultats précédents montrent que les caractéristiques physico-chimiques des amidons d'amylacés tropicaux varient en fonction du genre botanique, mais aussi de l'espèce et parfois des cultivars.

Chez les tubercules du genre *Dioscorea*, la variabilité notable des dimensions des grains d'amidon, des teneurs en amylose, des spectres de diffraction X, des sensibilités à l'alpha-amylase et des propriétés de gonflement dans l'eau, permet de discuter des relations éventuelles entre ces différents paramètres.

1. — La teneur en amylose semble en relation, pour certains amidons, avec la taille des grains. Chez les ignames, les amidons de *D. esculenta* et *D. dumetorum* possèdent à la fois les grains les plus petits et les teneurs en amylose les plus basses (Delpeuch *et al.*, 1978). La même observation peut être faite avec la variété de taro petite taille. Ces résultats vont d'ailleurs dans le même sens que ceux de Kulp (1973) pour l'amidon de blé.

2. — Les amidons d'ignames à petits grains et à faible teneur en amylose sont plus facilement hydrolysés par l'alpha-amylase que ceux des autres espèces. Ces résultats s'accordent avec ceux de Rasper (1969) qui, opérant avec des conditions différentes d'amylolyse (0,2 à 1 p. 100 d'enzyme par rapport au substrat,

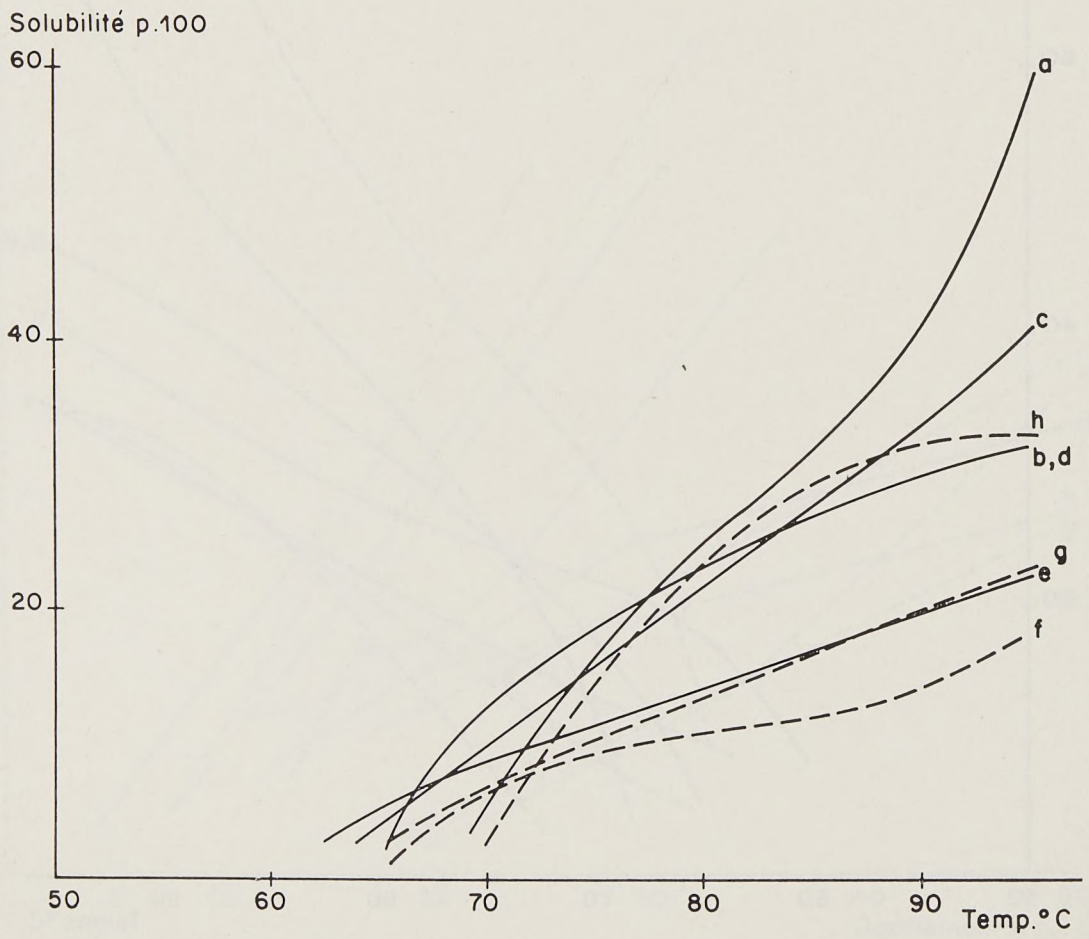

Fig. 7. — *Solubilité de divers amidons en fonction de la température*
a) *Colocasia* petite taille; b) *Colocasia blanc*; c) *Colocasia* rouge; d) *Xanthosoma* rouge; e) *Ipomea* jaune; f) *A. communis apyrena*; g) *A. communis seminifera*; h) *A. integra*.

durant 20 heures à 30 °C), trouve aussi que les amidons de *D. esculenta* et *D. dumetorum* sont plus dégradés que ceux des autres ignames. Dans l'ordre croissant de leur résistance à l'hydrolyse, il classe comme suit les amidons qu'il a examinés : taro, maïs, D. *dumetorum*, manioc, *D. esculenta*, patate douce, macabo, *D. alata*, *D. rotundata*, *D. cayenensis*, pomme de terre. Seule apparaît une inversion entre manioc, *D. dumetorum* et taros, qui pourrait être attribuée aux conditions expérimentales différentes. Encore faut-il remarquer que si les amidons des taros dans notre expérimentation présentent des vitesses finales d'amylolyse et des quantités d'amidon dégradé au bout de 2 h inférieures à celles de l'amidon du manioc, ils ont par contre des vitesses initiales d'amylolyse sensiblement supérieures, ce qui rejoint les résultats de RASPER.

Pour cet auteur, cette sensibilité accrue à l'action de l'enzyme serait due pour l'essentiel à la petite taille des grains. Au contraire, pour LEACH et SCHOCH (1961) et pour MERCIER (1968), il n'existerait aucune corrélation entre la taille ou la surface des grains d'amidon et la sensibilité à l'alpha-amylase; par contre, l'origine botanique d'un amidon et sa teneur en amylose seraient impliquées dans les différences de sensibilité à l'alpha-amylase. MERCIER (1968) montre ainsi que, pour le maïs, la vitesse et l'importance de l'hydrolyse enzymatique diminuent

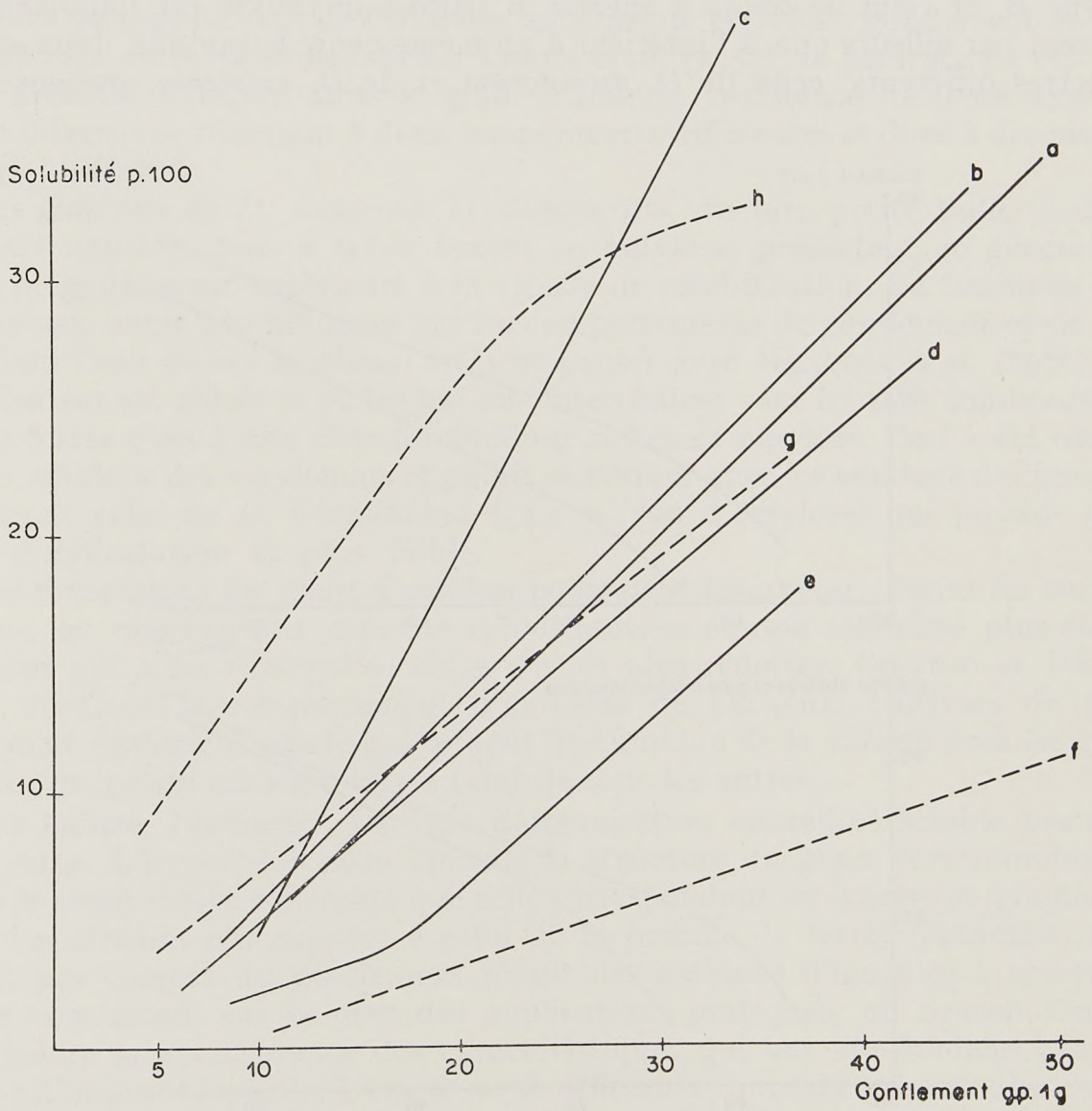

FIG. 8. — *Solubilité en fonction du gonflement de divers amidons*
a) *Colocasia* petite taille; b) *Colocasia* blanc; c) *Colocasia* rouge, d) *Xanthosoma* rouge, e) *Ipomea* jaune; f) *A. communis apyrena*; g) *A. communis seminifera*; h) *A. integra*.

avec l'augmentation de la teneur en amylose. Si comme le suggère BADENHUIZEN (cité par MERCIER, 1968) l'amylopectine est préférentiellement dégradée par les amylases, on pourrait penser que le rapport amylose /amylopectine a effectivement une influence sur l'alpha-amylolyse. Pourtant cette hypothèse ne se vérifie pas avec tous les genres botaniques puisque, pour *Artocarpus*, l'hydrolyse de l'amidon de la variété *seminifera* (21,8 p. 100 d'amylose) est aussi importante que celle de l'amidon de l'*A. apyrena* (9,2 p. 100 d'amylose).

L'hypothèse d'une corrélation entre le type du spectre de diffraction des rayons X du grain d'amidon, donc de son organisation cristalline, et l'alpha-amylolyse a été avancée par certains auteurs. Par exemple, LEACH et SCHOCH (1962) observent que les amidons à spectre A sont plus facilement attaqués par les amylases et se solubilisent plus rapidement dans le diméthylsulfoxyde que les amidons à spectre B. Pour ces auteurs, ceci suggèrerait l'existence d'une structure granulaire plus homogène chez les amidons à spectre B. Cette hypothèse s'accorderait avec le fait que les résidus d'hydrolyse acide de ces amidons sont à spectre B, alors que ceux des amidons de blé et de manioc n'ont plus tout à fait le même spectre A que les amidons natifs (DUPRAT *et al.*, 1974). Nos résultats, ainsi que ceux de BEWA (1978) sur le *canna* ne vérifient pas cette hypothèse puisqu'ils mettent en évidence que pour l'amidon de *D. esculenta*, à spectre très voisin du type B, et celui de *canna* à spectre B l'alpha-amylolyse est importante. Ils montrent par ailleurs que, à l'intérieur d'un même genre botanique, deux amidons à spectres différents, ceux de *D. dumetorum* et de *D. esculenta*, présentent des

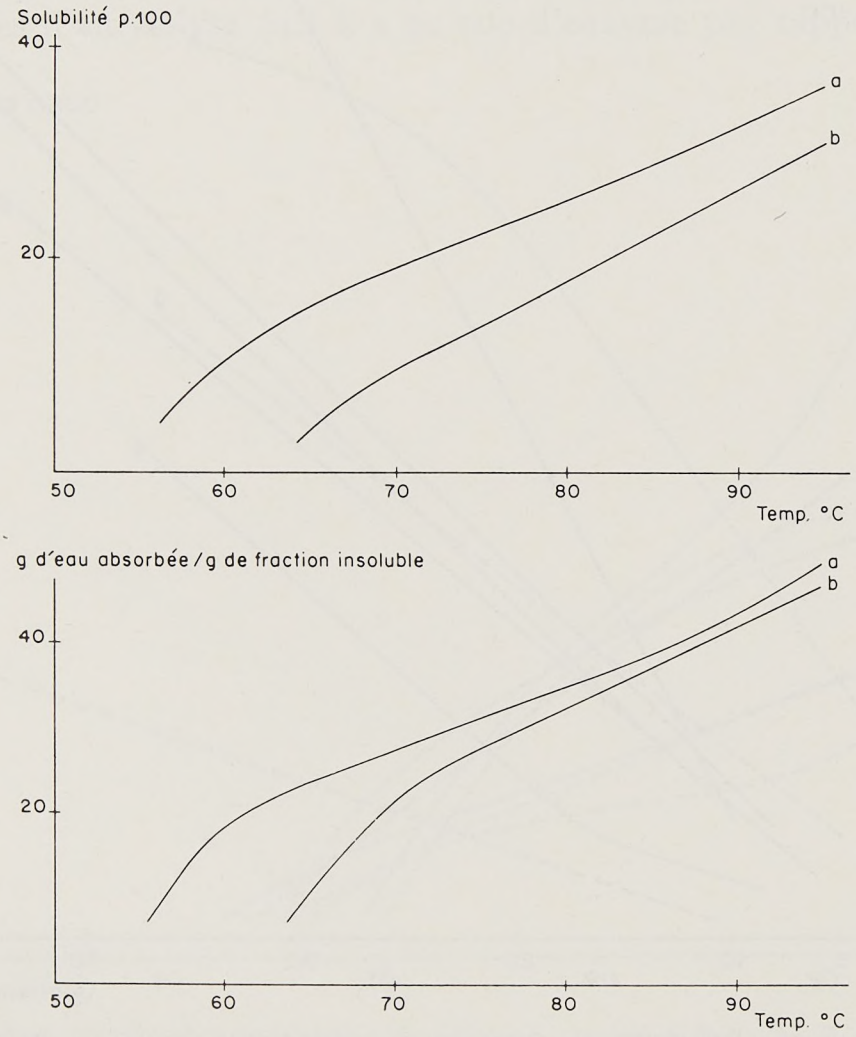

FIG. 9. — *Gonflement des amidons de Pachyrrhizus Erosus (a) et Coleus Esculentus (b) en fonction de la température*

sensibilités voisines et relativement élevées à l'action de l'alpha-amylase. Il semble donc que l'influence de l'organisation cristalline du grain d'amidon sur la sensibilité à l'alpha-amylase ne soit pas prépondérante.

En définitive, l'alpha-amylolyse importante des amidons à petits grains et à faible teneur en amylose indique que ces deux paramètres interviennent très probablement mais qu'il convient de rechercher ailleurs l'explication des différences de sensibilité entre les autres amidons. GUILBOT et MERCIER (1962) attribuent les variations de sensibilité des amidons aux amylases à l'existence de couches concentriques, autour du grain d'amidon, présentant des résistances différentes à la pénétration et à l'attaque enzymatique; l'organisation cristalline et/ou la teneur en amylose n'interviendraient que comme facteur secondaire. On pourrait ainsi proposer pour les grains d'amidons d'ignames un modèle identique à celui des grains d'amidon de pomme de terre avec une couche externe assez résistante limitant le processus d'alpha-amylolyse.

4. — Les allures comparables des diagrammes de solubilité et de gonflement de la plupart des amidons étudiés, en accord avec les résultats de HOLLO et GUILBOT (1964), indiquent une relation étroite entre les phénomènes de gonflement et de solubilité. Hormis le gonflement exceptionnel de l'amidon de *D. esculenta*, il semble que les caractéristiques de gonflement et de solubilité des amidons d'ignames se rapprochent de celles des amidons de céréales : gonflement et solubilité limités, en deux stades. Selon LEACH *et al.* (1959), la présence de ces deux stades pourrait indiquer dans le grain d'amidon l'existence de deux types de liaisons internes se relachant à deux températures différentes et donc à des niveaux d'énergie différents.

Les amidons de *D. esculenta*, *D. dumetorum*, du taro petite taille et de *A. communis apyrena*, tous à faible teneur en amylose, présentent en général une vitesse de gonflement supérieure à la vitesse de solubilisation. La teneur en amylose pourrait avoir une influence sur les comportements de gonflement et de solubilité dans l'eau de ces amidons; on peut penser avec MERCIER *et al.* (1970), que le gonflement est réduit là où les liaisons interchaînes sont les plus nombreuses ou les plus fortes c'est-à-dire chez les amidons riches en amylose. Ceci a été observé avec les amidons des amylomaïs et parait se vérifier avec les amidons des ignames, notamment celui de *D. libreschiana* (34,5 p. 100 d'amylose) qui possède la capacité d'hydratation la plus faible.

Les dimensions du grain d'amidon pourraient intervenir. Parmi les amidons de taros, on observe une capacité d'hydratation et une solubilité plus élevées chez celui qui a les dimensions du grain les plus réduites. GŒRING et DEHARS (1972), étudiant le comportement d'amidons de plusieurs cultivars de taros, remarquent également que le gonflement de l'amidon de la variété dont les grains sont les plus petits est supérieur à celui de tous les autres.

Par contre, l'influence du type d'organisation cristalline semble beaucoup moins nette. L'hypothèse selon laquelle la structure du grain correspondant au spectre B serait moins cohérente que celle correspondant au spectre A (gonflement limité des céréales par rapport à celui de la pomme de terre) (MERCIER, 1968) ne rend pas compte du gonflement réduit des amidons d'ignames à spectre B.

En conclusion, sur le plan des applications pratiques, on conçoit l'intérêt que présente la connaissance des caractéristiques qui ont été étudiées chez ces amidons d'origine tropicale. L'existence de différences importantes entre les espèces et les variétés peut conduire à choisir, dans le cadre du développement de la culture industrielle des tubercules, celles qui sont les plus adaptées à la cuisson, à certaines technologies ou à l'alimentation animale. C'est ainsi qu'en raison de la plus grande

sensibilité à l'hydrolyse alpha-amylasique de leurs amidons, l'utilisation des tubercules crus de *pachyrrhizus, D. dumetorum, D. esculenta* et *colocasia* blanc pourrait être envisagée en alimentation animale. CERIGHELLI (1955) avait déjà signalé *pachyrrhizus* comme légumineuse à tubercule susceptible de fournir de la fécule. Elle serait en outre facile à traiter du fait qu'elle est dépourvue de matières mucilagineuses. Si l'on recherche la propriété de cuire rapidement, *D. esculenta* sera choisi de préférence à *D. bulbifera* et à plus forte raison à *D. libreschiana* car son amidon, comme les amidons de pomme de terre et de manioc, gonfle facilement dans l'eau quand la température augmente alors que l'amidon de *D. libreschiana* et à un moindre degré celui de *D. bulbifera*, gonflent plus difficilement, comme celui du maïs par exemple.

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Summary

Characteristics of starches from tropical food plants: alpha-amylase hydrolysis, swelling and solubility patterns

Alpha-amylase hydrolysis, swelling and solubility patterns were studied on thirty starches from tropical plants of various genus, species and varieties. Large differences were observed in alpha-amylase hydrolysis, swelling and solubility patterns between the different genus and species and at times between varieties of the same species. The results were compared with those obtained with maize starch and potato starch. Relationships between the characteristics studied and the size of starch granules, the amylose content and the crystalline organization type were discussed.

Generally it seems that starches with small granules and with a low amylose content are more sensitive to alpha-amylase action than the other starches and have the lowest solubilization rate when compared to the swelling rate.

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Conséquences des processus technologiques de transformation des viandes de porc mâle sur la teneur en androsténone des graisses

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Résumé

Les viandes de vingt-neuf jeunes porcs mâles entiers sont transformées individuellement en jambons cuits ou en saucissons secs. La teneur en androsténone des graisses est mesurée sur les viandes fraîches et sur les produits transformés. La teneur moyenne en ce stéroïde diminue davantage après une cuisson (— 46 p. 100 de la teneur initiale pour le jambon cuit) qu'après une maturation (— 23 p. 100 pour le saucisson sec). La réduction de la concentration en androsténone étant reliée positivement à la teneur initiale ($r = +0.89$ à $+0.93$), on observe une diminution de la variabilité des concentrations en ce stéroïde. La disparition partielle d'androsténone au cours des fabrications est ainsi un élément favorable à une meilleure acceptabilité des viandes de porc mâle entier après transformation en produits de charcuterie.

Mots clés : *androsténone, odeurs sexuelles, porc mâle entier, transformation des viandes.*

Introduction

Les viandes de certains porcs mâles non castrés peuvent présenter au cours de la cuisson des odeurs désagréables dites « odeurs sexuelles », dues au stockage dans les graisses d'un composé à odeur urinaire : l'androsténone (5α androst-16-ène-3-one, stéroïde isolé par PATTERSON, 1968). Lors de la consommation en frais, ces défauts d'odeurs entraînent une réduction notable de l'acceptabilité des viandes par les consommateurs par comparaison à celles des témoins mâles castrés ou femelles (DESMOULIN et RHODES, 1975; BONNEAU, DESMOULIN et DUMONT, 1979).

La possibilité d'utiliser les viandes mâles présentant des défauts d'odeurs sexuelles pour la fabrication de certains produits transformés à d'abord été établie par PEARSON *et al.* (1971). Par la suite l'importance du mode de consommation (MAARSE *et al.*, 1972) et l'influence d'additifs de fabrication qui peuvent contribuer à masquer les odeurs et les goûts désagréables (PLIMPTON *et al.*, 1977) ont été mises en évidence. Dans cette étude nous avons voulu voir quel est le devenir de l'androsténone au cours des processus de transformation. Deux produits (jambon cuit et saucisson sec) correspondant à deux procédés technologiques très différents (saumurage-cuisson et séchage-maturation) ont été étudiés.

Matériel et méthodes

Cinquante quatre jeunes porcs mâles entiers de race Large White ont été abattus à 95 kg (34 animaux) ou 107 kg (20 animaux) de poids vif. La teneur en androsténone du tissu adipeux dorsal a été mesurée à l'aide de la méthode de dosage radioimmunologique mise au point par CLAUS (1974); les résultats sont exprimés en microgrammes d'androsténone par gramme de lipides. La sensibilité du dosage étant de 0,1 µg/g, sa reproductibilité est de 11 p. 100 (cœfficient de variation des mesures sur un échantillon témoin dosé au cours de chaque série d'analyses.) Compte tenu de ces limites de sensibilité et de reproductibilité du dosage, la mise en évidence d'une diminution de la concentration en androsténone serait peu fiable dans le cas des teneurs initiales faibles. C'est pourquoi, seuls vingt neuf porcs, qui offraient une teneur supérieure à 0,5 µg/g ont été retenus pour les opérations de transformation réalisées individuellement pour chaque animal. Le jambon a été transformé en jambon cuit et la fabrication du saucisson sec a été réalisée à partir de muscles et de graisses des régions antérieure et dorso-lombaire de la carcasse. La teneur en androsténone a été alors mesurée sur un échantillon de graisses prélevé sur chacune des fabrications. Les différences de teneur en androsténone entre les divers tissus gras de la carcasse étant assez peu élevées (CLAUS 1975, ANDRESEN et BAKKE, 1975), on peut considérer que les concentrations mesurées sur le tissu gras dorsal représentent approximativement la situation du stockage du stéroïde au niveau des matières premières de fabrication.

Résultats et discussion

La teneur en androsténone des graisses dorsales des vingt neuf porcs mâles étudiés s'élève en moyenne à 1,21 µg/g (tabl. 1). La distribution des individus selon les teneurs mesurées est rapportée à la figure 1. Dans le cas de viandes consommées en frais, cette gamme de concentrations correspond à des odeurs sexuelles peu prononcées en dessous de 1 µg/g et à des défauts marqués au-dessus de cette valeur (DESMOULIN et BONNEAU, résultats non publiés).

La teneur en androsténone des graisses des jambons cuits s'élève en moyenne à 0,56 µg/g, ce qui correspond à une diminution de 0,65 µg/g, soit 45,9 p. 100 de la concentration initiale. Dans le cas du saucisson sec la teneur moyenne des graisses est de 0,74 µg/g; la diminution de concentration (0,47 µg/g soit 23,0 p. 100 de la teneur initiale) est en moyenne moins importante que pour le jambon cuit.

TABLEAU I

Variations de la teneur en androsténone des graisses après transformations

	Teneur en Androsténone (µg /g)	Diminution de la teneur en androsténone	
		en valeur absolue (µg /g)	en valeur relative (%)
Tissu gras dorsal			
Moyenne	1.21		
Coefficient de variation. .	84 %		
Jambon cuit			
Moyenne	0.56	0.65	45.9
Coefficient de variation. .	70 %	131 %	40 %
Saucisson sec			
Moyenne	0.74	0.47	23.0
Coefficient de variation. .	63 %	197 %	142 %

La diminution de la teneur en androsténone est étroitement liée à la concentration initiale en ce stéroïde, notamment dans le cas du jambon cuit. Si Y est la diminution de teneur (µg /g) et X la teneur initiale (µg /g), on a en effet :

— *dans le jambon :*

$$Y = 0.7770\ X - 0.2890; \quad r = +\ 0.926 \quad P < 0.001$$

— *dans le saucisson :*

$$Y = 0.8082\ X - 0.5086; \quad r = +\ 0.888 \quad P < 0.001$$

Le degré de liaison entre Y et X est amélioré de façon significative ($P < 0.05$) en établissant les régressions quadratiques suivantes :

$$Y = 0.0948\ X^2 + 0.3304\ X + 0.0184; \quad R^2 = 0.889 \text{ pour le jambon}$$

$$Y = 0.1098\ X^2 + 0.2908\ X - 0.1526; \quad R^2 = 0.826 \text{ pour le saucisson}$$

Ainsi, 82,6 à 88,9 p. 100 de la variabilité de la diminution de teneur en androsténone est expliquée par les variations de la concentration initiale.

La réduction de teneur en androsténone étant ainsi reliée positivement à la concentration initiale, les fortes teneurs sont réduites de façon plus importante que les faibles en valeur absolue; c'est pourquoi la variabilité est un peu plus faible après transformation. Celle-ci reste néanmoins importante (C.V. de 70 et 63 p. 100) car, d'une manière générale, les viandes ayant les teneurs les plus fortes conduisent aux produits ayant les concentrations les plus élevées. En effet, le

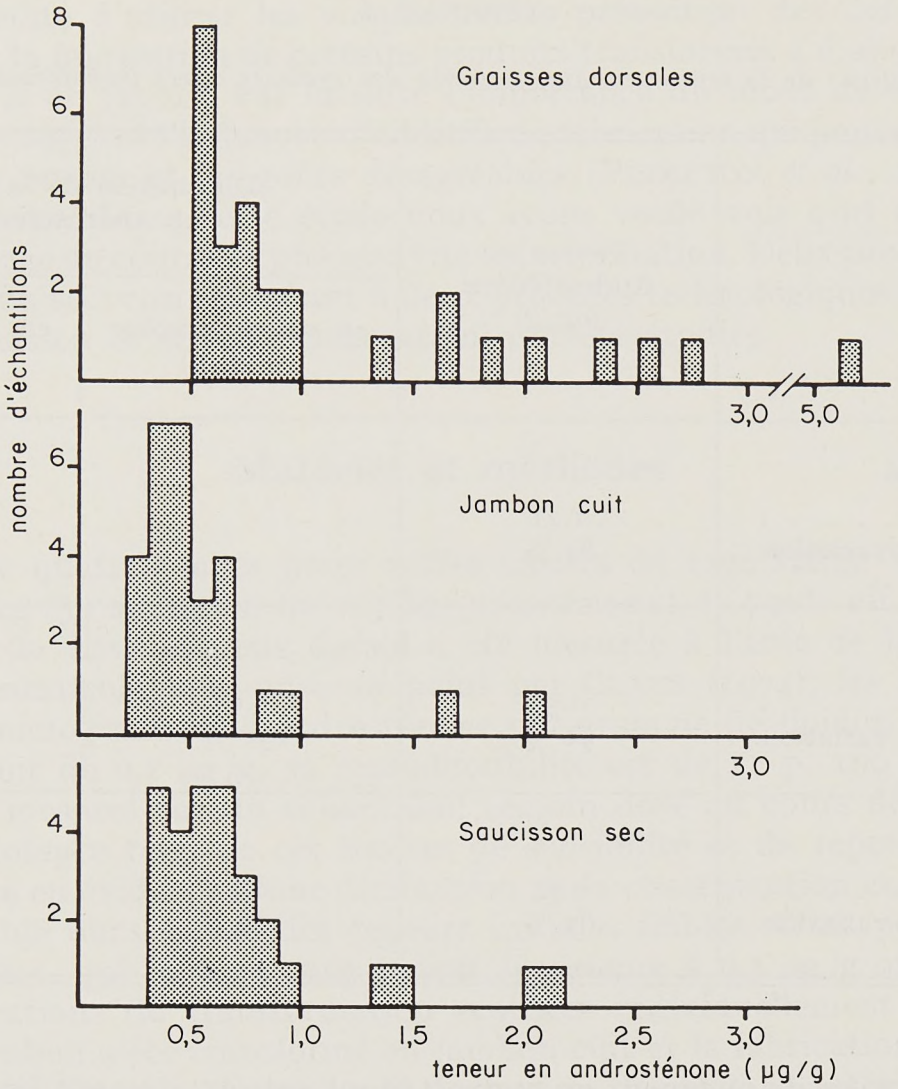

FIG. 1. — *Distribution des teneurs en androsténone des graisses*

cœfficient de corrélation entre les teneurs mesurées sur le tissu gras dorsal et celles mesurées sur les produits s'élève à + 0.575 ($P < 0.01$) pour le jambon et à + 0.417 ($P < 0.05$) pour le saucisson.

Dans le cas du jambon cuit, la disparition d'une partie de l'androsténone pourrait être expliquée par une volatilisation au cours de la cuisson. Une dissociation du composé semble devoir être exclue car ce stéroïde, qui fond à 135 °C, est stable jusqu'à des températures bien supérieures à celles utilisées pour la cuisson des jambons. La réduction de la concentration en androsténone au cours de la fabrication du saucisson sec reste peu expliquée. Elle est probablement due aux processus de fermentation microbienne au cours de la maturation. Dans ce cas, l'hypothèse d'une formation éventuelle d'autres composés malodorants, par exemple la réduction de l'androsténone en androsténol à odeur de musc, ne peut pas être exclue.

La disparition partielle d'androsténone aux cours des fabrications est un élément favorable à la réduction des défauts d'odeurs sexuelles des viandes de porc mâle. Les résultats des enquêtes d'acceptabilité auprès des consommateurs et des analyses sensorielles réalisées par un jury de laboratoire nous permettront d'établir l'influence des résidus d'androsténone sur l'acceptabilité de ces produits transformés.

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Summary

The effect of processing boar meat on androstenone concentration in fat

Cooked hams and dry sausages have been processed individually from pork of 29 young boars. Fat androstenone concentration has been measured by radioimmunoassay first in pork and then in hams and sausages. Mean androstenone concentration is more reduced after cooking (— 46 p. 100 of initial content for ham) than after maturation (— 23 p. 100 for dry sausage). As decrease of androstenone content is positively related to initial concentration ($r = + 0.89$ to $+ 0.93$) the variability of steroid measurements is reduced after processing. So, partial disappearance of androstenone is propitious to a better acceptability of boar meat after processing.

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Analyse automatique d'enregistrements de chromatogrammes

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Résumé

La saisie des données en temps réel et le traitement automatique des renseignements provenant d'un analyseur d'acides aminés ont été obtenus en reliant par câble cet appareil à un ordinateur MITRA 15.

L'enregistrement sur cassettes peut remplacer la ligne directe si le traitement différé est acceptable.

Des méthodes de traitement des données avec modélisation du système ont été mise au point. La quantification d'une part importante des erreurs d'expérimentation a été réalisée. En outre, la dissociation de pics partiellement confondus dans les chromatogrammes a été obtenue.

Mots clés : *Acquisition données, temps réel, acides aminés, chromatographie, ajustement non linéaire, modélisation.*

Introduction

L'automatisation des techniques d'analyse des séparations chromatographiques soulève de nombreux problèmes qui peuvent être classés en deux groupes.

1. — Problèmes liés à l'acquisition des données : saisie automatique d'enregistrement des chromatogrammes, transmission en temps réel ou de façon différée de ces informations...;

2. — Problèmes relatifs aux traitements des informations recueillies, contrôles permettant de fournir des « données propres », analyse de ces données de façon la plus exhaustive pour en extraire tous les renseignements qu'elles contiennent.

La saisie en elle-même demande à la fois d'utiliser correctement un matériel d'analyses physico-chimiques parfois vétuste et de retenir les informations les plus pertinentes. Il conviendrait, par exemple, de choisir le type de scrutation (fréquence d'échantillonnage, filtrage, échantillonnage sélectif) qui assure la conservation des seules données utiles.

Le mode de transmission doit dépendre de l'objectif poursuivi et des contraintes que cet objectif implique. Est-il nécessaire d'employer la transmission en temps réel ou au contraire de mettre les informations sur un support comme les cassettes pour les traiter de façon différée? Dans certains cas un contrôle de l'expérimentation est nécessaire. Ainsi une partie des données recueillies jusqu'à l'instant t peut servir à modifier l'évolution ultérieure de la manipulation. La solution en temps réel s'impose donc; il n'en est plus de même si le contrôle n'est d'aucun intérêt véritable.

Le traitement des données peut se faire en déterminant de façon plus ou moins aveugle les surfaces des pics des chromatogrammes. Ces surfaces sont les éléments de base pour calculer les quantités de substrats séparés; cette connaissance représente le but principal des manipulations.

La façon de calculer les surfaces n'est pas « neutre » dans les interprétations que l'opérateur en fait.

La solution « clé en main » proposée par de nombreux fournisseurs de matériel d'analyse des données n'est guère raisonnable dès que l'utilisateur souhaite maîtriser l'ensemble des éléments de la chaîne. En particulier la connaissance de la méthode d'intégration est un élément capital pour que l'utilisateur sache parfaitement ce qu'il calcule.

La détermination de la précision des surfaces n'est généralement pas calculable par les méthodes classiques. Nous n'arrivons à l'estimer qu'en répétant les manipulations un certain nombre de fois. En revanche, si nous pouvons fournir un modèle analytique de la forme des pics, il est alors possible de donner pour une seule séparation chromatographique une estimation de la précision tant des positions que des surfaces.

Chacun des problèmes est en soi relativement limité et ne demande pas un niveau d'approche particulièrement élevé; par contre, le «système global » est beaucoup plus délicat à maîtriser. En outre, l'étude de chaque élément de ce système fait apparaître des questions nouvelles. Sans tomber dans le perfectionnisme, il semble nécessaire de fournir des réponses à ces questions plus satisfaisantes que celles apportées actuellement.

Voici 3 ans, des travaux ont été entrepris afin de traiter sur l'ordinateur MITRA 15 de la Station de Biométrie de Montfavet, les données obtenues à partir d'un analyseur d'acides aminés Biocal BC200 (Type 1967).

La proximité relative (200 mètres) des 2 appareils nous laissait entrevoir la possibilité de pallier, à moindre coût, l'absence de calculateur associé au BC200.

L'objet de cet article est de faire le point sur les problèmes qui sont apparus, sur les résultats actuels des travaux et sur les orientations futures des recherches.

Méthodes et techniques

Nous avons voulu comparer les résultats d'un traitement manuel des données et ceux obtenus par des traitements automatiques.

2.1. — *Procédé Manuel*

La mesure quantitative d'un acide aminé est proportionnelle à la surface de son pic d'élution.

Pour calculer cette surface, nous utilisons un procédé simplifié (fig. 1*a*) qui « consiste à assimiler le pic à un triangle dont nous mesurons la surface en multipliant sa hauteur par sa largeur à la demi-hauteur » (JAULMES, 1963).

Cette surface ne représente que 94 p. 100 de la surface réelle. L'erreur systématique qui découle d'une telle approximation est partiellement corrigée par le mode de calcul des cœfficients de coloration. En effet, les cœfficients sont déterminés par comparaison avec les pics obtenus à partir de quantités connues des acides aminés correspondants.

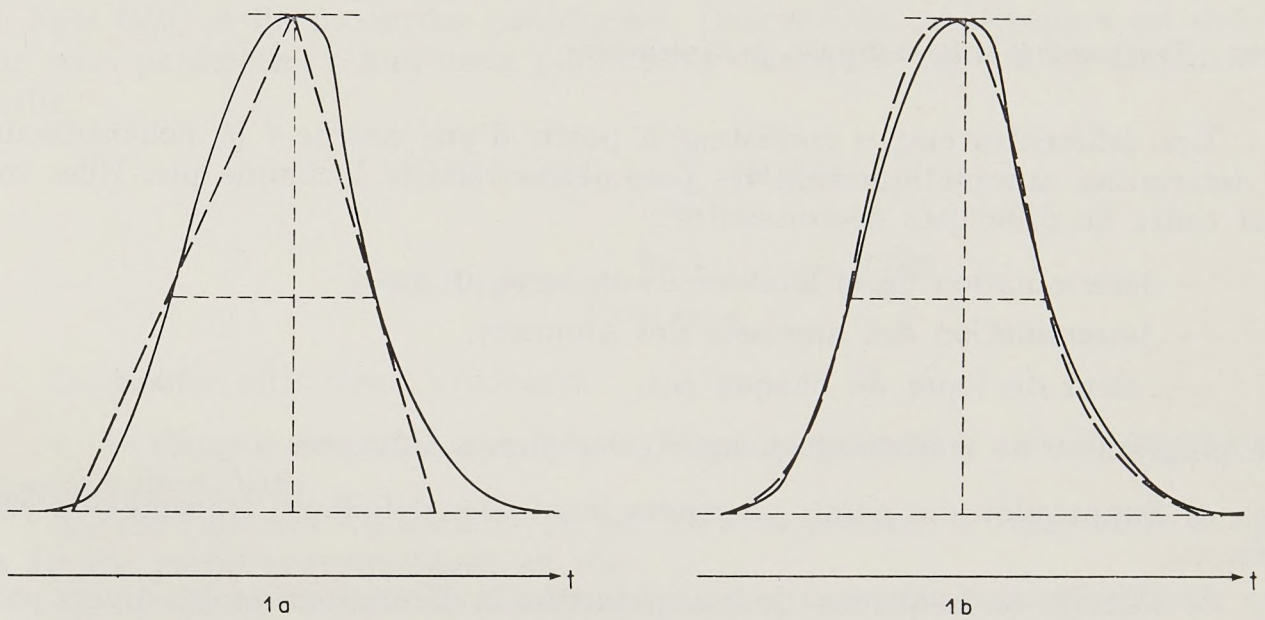

FIG. 1. — *Calcul de la surface d'un pic*

a) par la méthode du triangle.
b) par analogie avec une gaussienne.

2.2. — *Procédés automatiques*

2.21. *Saisie automatique*

L'analyseur d'acides aminés fournit un signal 0-50 mv qu'il faut amplifier puis convertir en numérique.

Le schéma de l'ensemble est représenté sur la figure 2.

Le convertisseur numérique code la donnée sur 10 bits soit une précision de 0,1 p. 100. La période de scrutation est de 1 seconde. L'acquisition se fait en temps réel. Dans cette première phase, les données reçues sont simplement stockées sur disque, le traitement scientifique se fait une fois l'acquisition complètement terminée.

Ce type de saisie a été adopté pour des raisons de disponibilité de l'ordinateur, de faible coût, sans oublier son intérêt technique au cours de sa mise au point.

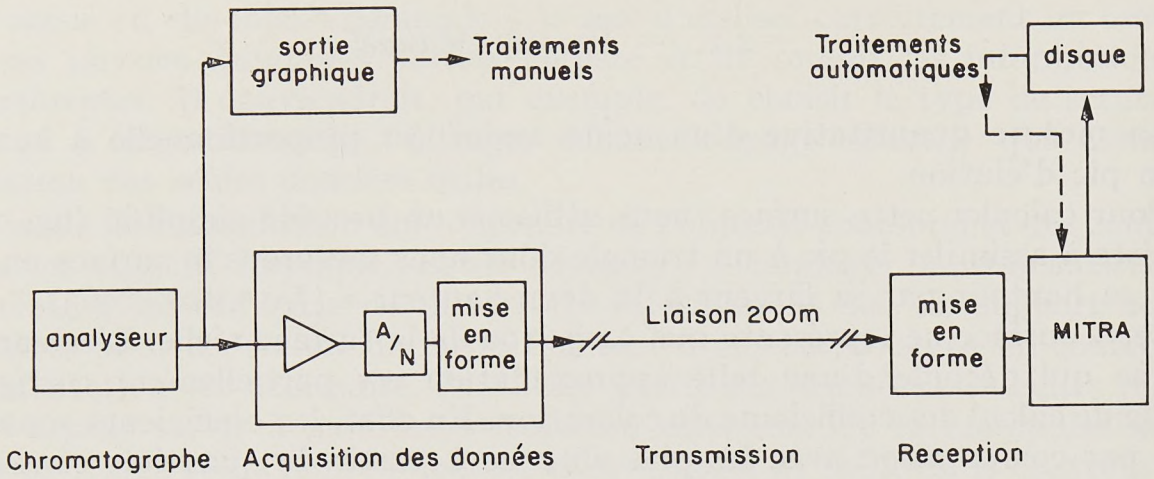

FIG. 2. — *Schéma de l'acquisition de données*

2.22. *Traitements automatiques préliminaires*

Les différentes étapes consistent à partir d'une courbe $f(t)$ échantillonnée à déterminer automatiquement les paramètres relatifs à chaque pic. Elles sont par ordre de difficultés décroissantes :

— détermination de la hauteur de la ligne de base;

— détermination des abscisses des sommets;

— calcul de l'aire de chaque pic.

Ce programme de traitement comporte les phases suivantes :

a) Suppression des points aberrants (analyseur défaillant, transmission erronée...).

b) Filtrage en fréquence. Ce lissage facilite la détermination des divers paramètres des pics.

c) Calcul des paramètres dans l'ordre indiqué ci-dessus. Ce calcul se rapproche beaucoup de la méthode manuelle à la différence que le triangle est remplacé par une gaussienne calculée sur trois points (fig. 1*b*).

Les valeurs trouvées servent de première estimation pour la mise en œuvre des traitements approfondis.

Les deux procédés décrits (manuel et automatique) présentent des limites. D'une part, la précision des résultats ne peut pas être estimée. D'autre part, ces procédés ne permettent pas de connaître les surfaces respectives de deux pics insuffisamment séparés puisque les calculs sont basés sur la largeur des pics à leur demi-hauteur.

2.23. *Traitements approfondis*

Nous avons donc été amenés à utiliser une autre technique de manière à tenter d'évaluer la surface de tous les pics même s'ils sont mélangés. En outre, nous cherchons à donner les intervalles de confiance sur les surfaces sans avoir à répéter plusieurs fois la même expérience.

Pour cela nous construisons un modèle paramétrique du phénomène.

$$Y(t) = f(\theta_1, \theta_2, \ldots \theta r; t) + \text{erreur}$$

où θ_1, θ_2,.....θr sont des paramètres à estimer. Pour faire cette estimation nous disposons de n observations Y_1, Y_2,....Y_n faites aux temps t_1, t_2...t_n; l'estimation consiste à rendre minimum la quantité :

$$S = \sum_{i=1}^{n} (Y_i - \hat{Y}_i)^2$$

où $\hat{Y}_i = f(\theta, t_i)$; nous notons θ sans indice pour représenter l'ensemble des r paramètres θ_k).

Ce critère des moindres carrés est employé par analogie avec ce qui se fait dans les modèles de régression non linéaire.

Le problème essentiel est de trouver une forme analytique satisfaisante de $f(\theta, t)$.

Si les pics sont symétriques, nous pouvons considérer en première approximation que l'ensemble des pics du chromatogramme est la somme d'une ligne de base $G(t)$, et de p courbes gaussiennes. Chacune de ces dernières est définie par trois paramètres : aux deux paramètres classiques s'ajoute un facteur d'échelle.

$$\hat{Y} = G(t) + \sum_{k=1}^{p} (H_k(t))$$

$$\text{avec } H_k(t) = \frac{\theta_{3k-2}}{\sqrt{2\pi}.\,\theta_{3k-1}} e^{-\frac{1}{2}\left(\frac{t-\theta_{3k}}{\theta_{3k-1}}\right)}$$

Ce modèle offre deux avantages :

1. — Chacun des trois paramètres d'une gaussienne a une interprétation physique immédiate.

θ_{3k} est l'abscisse du sommet du pic. Il nous permet de déterminer le nom de l'acide aminé correspondant au pic.

θ_{3k-1} est une mesure de l'étalement du pic; il est d'autant plus faible que la colonne a un bon pouvoir séparateur mais il dépend ausssi de la solution tampon utilisée et de l'acide aminé correspondant au pic.

θ_{3k-2} facteur d'échelle, est la surface de la courbe : c'est le paramètre qui nous intéresse directement.

2. — Il n'est pas nécessaire d'évaluer simultanément les paramètres des gaussiennes correspondant à tous les pics du chromatogramme. En effet, quand on s'éloigne du sommet d'un pic, la courbe gaussienne correspondant à ce pic tend rapidement vers des valeurs négligeables; dans ces conditions, l'influence du reste du chromatogramme sur un pic isolé est insignifiante. Nous dissocions donc l'ensemble du chromatogramme en blocs indépendants comprenant un pic s'il est suffisamment isolé (fig. 3; bloc 1) ou plusieurs pics mélangés (fig. 3; bloc 2).

Au bloc 1, correspond les 3 paramètres d'une gaussienne.

Au bloc 2, correspondent les 6 paramètres de deux gaussiennes :

$$H_1 + H_2 = \frac{\theta_1}{\sqrt{2\pi}.\,\theta_2} e^{-\frac{1}{2}\left(\frac{t-\theta_3}{\theta_2}\right)^2} + \frac{\theta_4}{\sqrt{2\pi}.\,\theta_5} e^{-\frac{1}{2}\left(\frac{t-\theta_6}{\theta_5}\right)^2}$$

Sur un bloc nous pouvons considérer que la ligne de base est soit constante, soit fonction linéaire du temps. Nous prendrons donc, pour chaque bloc, une fonction $G(t) = \theta_{3p+1}$ ou une fonction $G(t) = \theta_{3p+1} \times t + \theta_{3p+2}$.

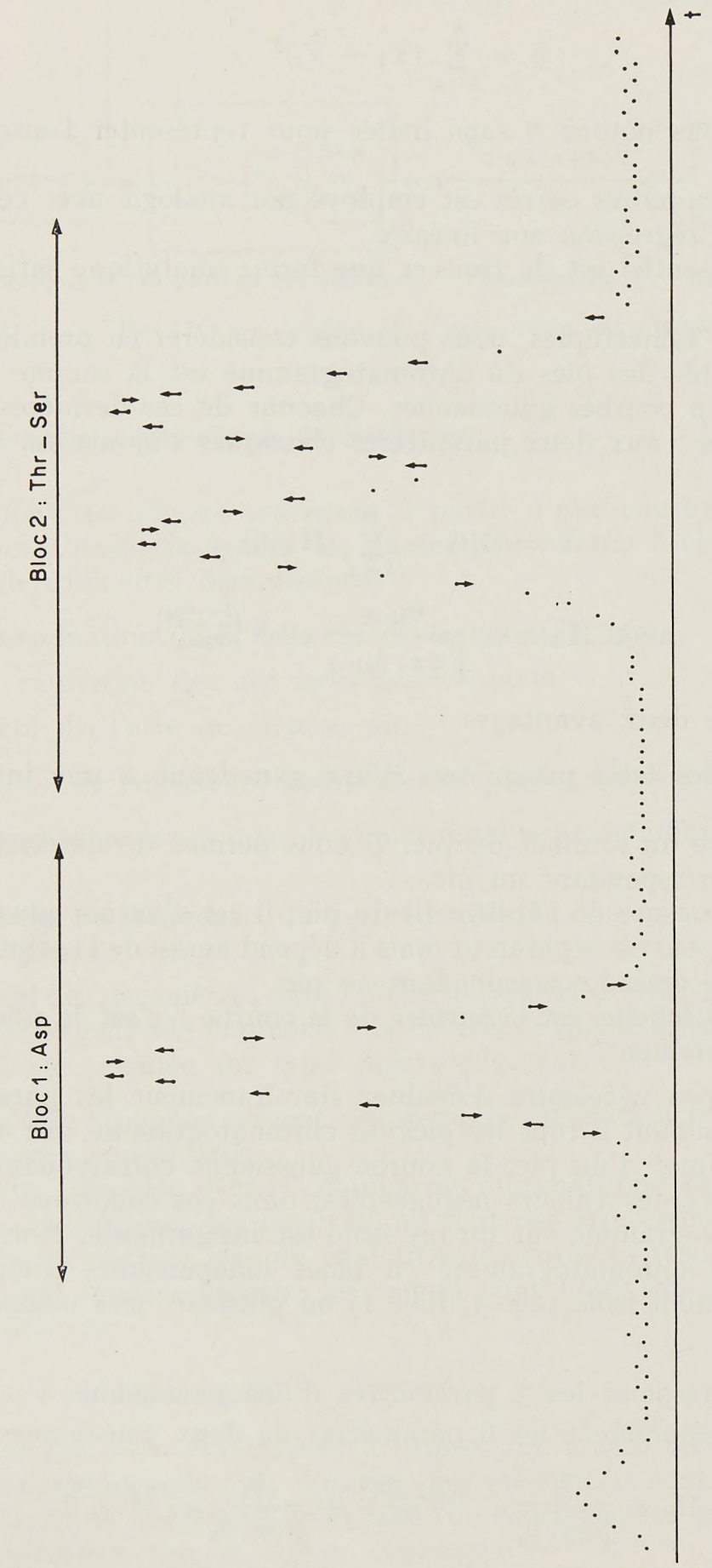

FIG. 3. — *Ajustement d'un modèle à la courbe expérimentale*

..... points expérimentaux
↑ résidus

Les techniques d'ajustement non-linéaire nous permettent d'estimer pour chaque bloc :

1 — les paramètres θ;

2 — les intervalles de confiance asymptotiques des paramètres;

3 — les cœfficients de corrélations entre les paramètres estimés. Ces derniers permettent de voir que les estimations ne sont pas indépendantes; des matrices de corrélations typiques sont présentées dans le Tableau 3.

Pour aboutir aux résultats, les programmes d'ajustement non linéaire procèdent par itérations, à partir d'estimations initiales des paramètres. Le nombre d'itérations (donc le temps de calcul) et la cohérence des résultats dépendent du choix de ces estimations. Les valeurs estimées fournies par la méthode automatique se sont généralement révélées satisfaisantes. Le traitement approfondi a permis simplement d'affiner les estimations. Le dépouillement d'un chromatogramme d'une vingtaine d'acides aminés dure entre une heure et une heure trente dans nos conditions expérimentales actuelles, lissage inclus.

Si l'estimation des paramètres ne soulève pas de problèmes très délicats (il suffit d'avoir un bon programme d'ajustement non linéaire), par contre les intervalles de confiance qu'il est possible de calculer ne sont valables que sous trois conditions assez restrictives : indépendance. normalité et égalité des variances des termes d'erreur. La condition d'indépendance est sans conteste la plus difficile à vérifier; l'analyse des résidus est un excellent instrument pour s'en assurer.

C'est sûrement dans cette direction qu'un effort théorique est à réaliser. En particulier, l'emploi de fonctions spline n'a pas encore été tenté; il est vraisemblable que leur application à ce type de données doit s'avérer fructueuse (Lawton, Sylvestre et Maggio, 1972; Shœnfeld et Devœ, 1976; Metzler *et al.*, 1977).

En conclusion, la méthode approfondie permet d'obtenir des résultats plus complets que les techniques traditionnelles. Néanmoins, cette méthode est basée sur des hypothèses relativement fortes qu'il convient de vérifier afin d'atteindre un degré de confiance suffisant au niveau des résultats; ces hypothèses étant surtout fortes quand nous voulons estimer des intervalles de confiance pour les paramètres. Nous parlerons plus loin de leur importance.

Résultats et discussion

Comparaison des trois méthodes d'analyse

Les résultats obtenus par les trois méthodes à partir de séparations chromatographiques successives d'une même solution synthétique étalon sont présentées dans le tableau 1.

Pour certaines séparations, des défauts de transmission n'ont pas permis l'analyse de l'ensemble des acides par les méthodes automatiques. Les quantités moyennes des acides sont donc calculées pour des nombres d'analyses différents selon les méthodes.

Dans l'état actuel du programme, il arrive que le traitement approfondi fournisse une évaluation aberrante de la ligne de base. Ceci nous a conduit à

TABLEAU I

Comparaison des résultats obtenus par les trois méthodes d'analyse

n = nombre d'analyses
$\bar{x}$ = valeur moyenne obtenue à partir des n mesures
τ = écart-type calculé à partir des n mesures

Méthode approfondie (non corrigée) = compte tenu de toutes les mesures
Méthode approfondie (corrigée) = mesures éliminées lorsque valeur ligne base apparemment surestimée

Méthode	Manuelle			Automatique			Approfondie (non corrigée)			Approfondie (corrigée)			$\frac{\bar{x} \text{ manuelle}}{\bar{x} \text{ approfondie corrigée}}$	$\frac{\bar{x} \text{ manuelle}}{\bar{x} \text{ automatique}}$
	n	$\bar{x}$	τ	n	$\bar{x}$	τ	n	$\bar{x}$	τ	n	$\bar{x}$	τ		
Acide Aspartique . . .	11	3,69	0,07	6	3,59	0,12	6	3,43	0,26	5	3,54	0,07	1,04	1,03
Thréonine	11	4,10	0.10	8	3,97	0,09	7	3,52	0,12				1,16	1,03
Sérine	11	4,20	0,09	8	4,14	0,12	7	3,76	0,21				1,12	1,01
Acide Glutamique. . .	11	3,35	0,08	8	3,29	0,19	8	3,09	0,23	6	3,21	0,09	1,04	1,02
Glycine	11	3,95	0,12	10	3,91	0,14	9	3,72	0,17				1,06	1,01
Alanine	11	4,12	0,07	10	4,13	0,12	10	3,71	0,40	6	3,95	0,16	1,04	1,00
Cystine	11	2,15	0,06	9	2,18	0,07	9	1,89	0,21	5	2,04	0,06	1,05	0,99
Acide β-amino-butyrique	11	4,41	0,14	9	4,29	0,11	9	4,12	0,12				1,07	1,03
Valine	11	4,11	0,05	10	4,08	0,13	10	3,74	0,22	8	3,82	0,16	1,08	1,01
Méthionine	11	3,95	0,10	10	4,01	0,32	10	3,62	0,13				1,09	0,99
Isoleucine	11	4,07	0,05	10	3,94	0,22	10	3,69	0,15				1,10	1,03
Leucine	11	3,98	0,08	10	3,78	0,47	9	3,65	0,08				1,09	1,05
Norleucine	11	4,79	0,20	10	4,70	0,17	10	4,46	0,18				1,07	1,02
Tyrosine	11	3,91	0,07	10	3,97	0,20	10	3,69	0,21				1,06	0,98
Phénylalanine	11	3,92	0,13	10	3,95	0,18	10	3,60	0,16				1,09	0,99
B-Alanine	11	2,67	0,31	9	2,83	0,36	9	2,47	0,54	6	2,59	0,38	1,03	0,94
Acide γ-amino-butyrique	11	4,91	0,21	9	4,81	0,23	8	4,60	0,23				1,07	1,02

supprimer un certain nombre de résultats pour le calcul de la moyenne et des écarts-types. Nous n'avons conservé dans la colonne « méthode approfondie corrigée » (tabl. 1) que les pics dont la ligne de base avait été estimée de manière satisfaisante.

La comparaison des moyennes et de leurs rapports montre que la méthode manuelle et la méthode automatique donnent des résultats du même ordre de grandeur (rapport 0,94 à 1,05).

La méthode « approfondie corrigée » conduit à des moyennes inférieures à celles obtenues en « manuel » (rapport 1,03 à 1,09). Le rapport est plus élevé pour la thréonine (1,16) et la sérine (1,12). Une explication de ce phénomène sera proposée dans le chapitre « Avantages de la méthode approfondie ». Les valeurs systématiquement plus faibles obtenues par cette dernière méthode sont en partie liées aux techniques de calcul et aux unités d'intégration; il semble cependant que la définition douteuse de la ligne de base joue un rôle important mais des solutions à ce problème sont envisagées.

La méthode manuelle conduit le plus souvent à des écarts types (calculés à partir des n mesures) légèrement plus faibles que ceux obtenus avec les méthodes automatiques.

Avantages de la méthode approfondie

Dans deux domaines, cette méthode apporte une finesse d'analyse plus grande que celle obtenue par les autres systèmes.

Lors d'une mauvaise séparation chromatographique d'un bloc de pics, (fig. 4), l'évaluation de la surface de chacun des pics par la méthode manuelle n'est pas

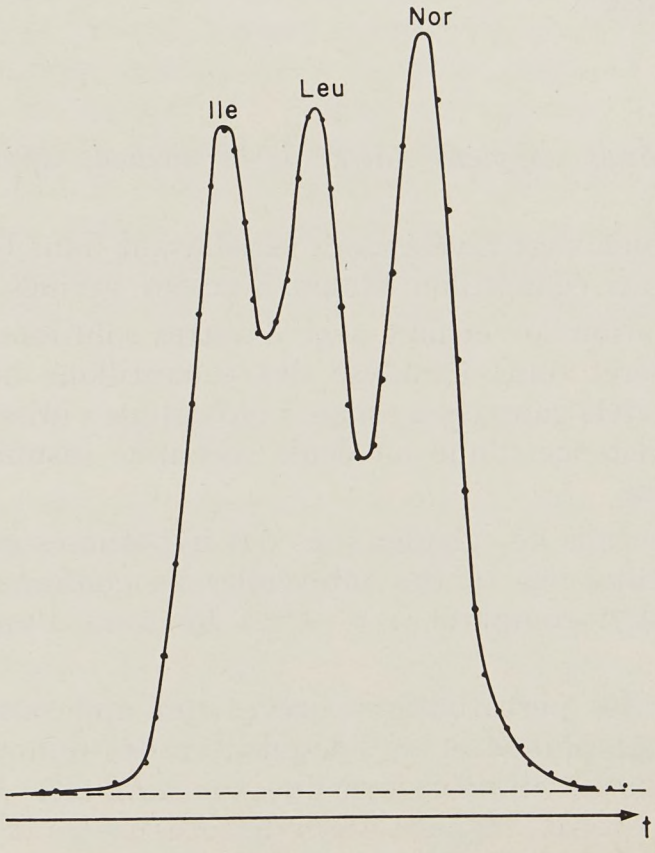

FIG. 4. — *Bloc de 3 pics mélangés*

possible. En revanche, la méthode approfondie permet de résoudre individuellement chaque pic. Cette méthode accepte donc un niveau de séparation chromatographique inférieur.

Les résolutions du mélange des deux pics thréonine-sérine par méthode manuelle donnent des valeurs par excès (fig. 5) car elles ne tiennent pas compte du recouvrement des courbes. La méthode approfondie évite un tel inconvénient. L'erreur de recouvrement est très importante dans l'évaluation d'un pic réduit accolé à un grand pic.

Ce problème de recouvrement n'est pas résolu par certains types d'intégrateurs.

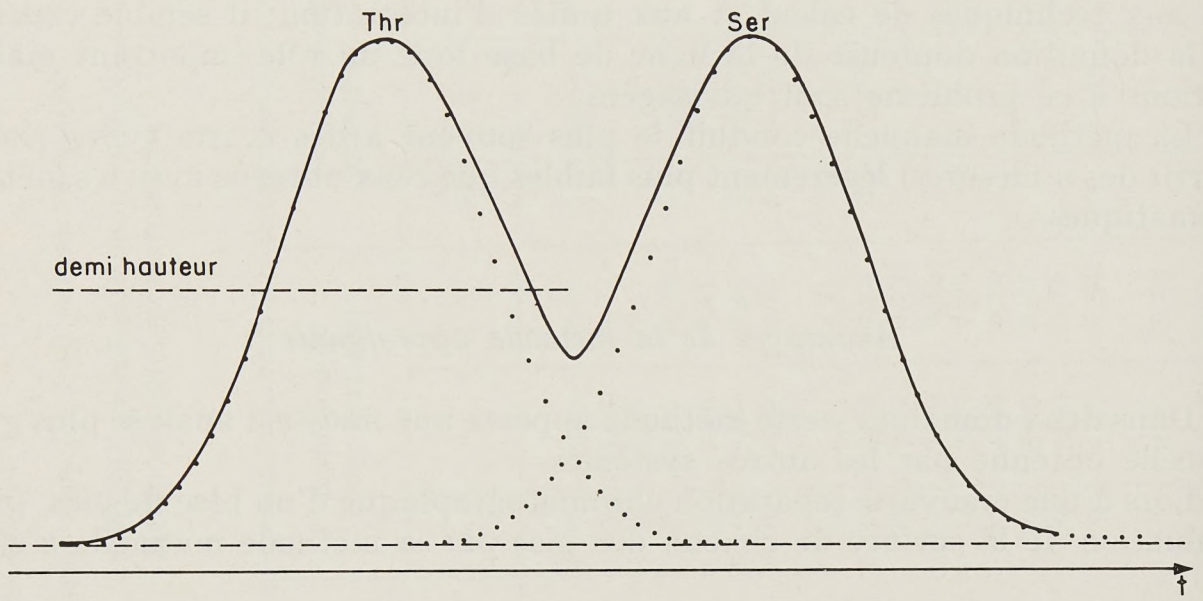

FIG. 5. — *Séparation d'une courbe en une somme de deux gaussiennes*
—— Courbe expérimentale
.... gaussiennes

Possibilités supplémentaires de la méthode approfondie

Le modèle gaussien s'est révélé assez satisfaisant dans l'analyse des résultats obtenus à partir d'un échantillon étalon d'acides aminés.

Si cette constatation se vérifiait avec d'autres solutions étalons, elle présenterait un grand intérêt dans l'analyse des échantillons biologiques. En effet, l'inadéquation du modèle gaussien à un pic « biologique » (dissymétrie par exemple) pourrait révéler l'existence d'une molécule inconnue insuffisamment séparée de l'acide aminé attendu.

La méthode a permis de calculer sous des hypothèses assez restrictives (voir le paragraphe « orientations »), des intervalles de confiance δ sur les surfaces (tabl. 2), où $\delta/4$ est à comparer à τ. δ est fonction d'une partie des sources d'erreurs.

Il doit intégrer les perturbations brèves qui apparaissent au cours de la séparation chromatographique et de l'acquisition des données. Par contre, δ ne prend pas en compte les imprécisions dues au contexte expérimental initial : mauvais ajustement du pH, régularisation défectueuse de la température, vieillissement de l'indicatif coloré, malfaçon dans le dépôt de l'échantillon...

TABLEAU 2

Comparaison des résultats obtenus par les 3 méthodes

Méthode	Manuelle	Automatique	Approfondie	
			Surface	$\delta/4$
Acide Aspartique	3,75	3,72	3,66	0,08
Thréonine	4,17	4,03	3,65	0,07
Serine	4,30	4,19	3,87	0,06
Acide Glutamique	3,42	3,35	3,28	0,14
Glycine	4,11	4,03	3,86	0,10
Alanine	4,20	4,16	3,95	0,09
Cystine	2,13	2,15	2,07	0,07
Valine	4,16	3,99	3,94	0,12
Méthionine	4,08	3,91	3,80	0,09
Isoleucine	4,12	3,98	3,52	0,18
Leucine	4,04	3,91	3,74	0,11
Tyrosine	3,96	3,81	3,72	0,05
Phénylalanine	4,00	3,85	3,70	0,06
Acide Amino 2 Butyrique	4,43	4,29	4,29	0,06
Norleucine	5,01	4,86	4,75	0,13

La méthode approfondie va donc nous donner une idée de la précision de la surface du pic pour une analyse donnée. δ est donc une information supplémentaire et précieuse par rapport au résultat obtenu par le système manuel.

Compte tenu du fait que l'intervalle de confiance ne dépend que d'une fraction des sources d'erreurs, le δ d'une mesure individuelle doit être sensiblement inférieur à 4τ (τ = écart type calculé sur n mesures; (tabl. 1).

En effet, lorsque les valeurs de δ sortent des normes, il convient de prendre les résultats avec prudence. L'analyse des résidus (fig. 3) est alors riche d'enseignements. S'il n'y a pas de tendance particulière des résidus, nous ne pouvons qu'accepter ce résultat malgré son imprécision. En revanche, si une tendance marquée apparaît, nous concluons que le modèle choisi n'est pas adapté. Il faudra donc envisager un autre modèle, dans la mesure du possible.

Orientations des recherches

Dans l'état actuel de nos recherches, de nombreuses améliorations semblent pouvoir être apportées à l'ensemble du système.

1. — Les traitements automatiques considèrent une fonction du temps échantillonnée sans aucune connaissance a priori de cette fonction. Or, dans des conditions expérimentales semblables, les acides aminés apparaissent toujours dans le même ordre et à des instants variant peu dans le temps.

Ces remarques devraient faciliter la détermination automatique des abscisses des sommets et l'identification des acides aminés correspondants.

2. — Tous les résultats sont conditionnés par *l'hypothèse : le modèle choisi est juste*. Il importe donc de vérifier l'adéquation du modèle aux données. Pour

TABLEAU 3

Matrices de corrélations

3*a* — Bloc 1 — Acide Aspartique : 3 paramètres.
3*b* — Bloc 2 — Thréonine — Sérine : 6 paramètres.

A = Surface du pic.
B = Abcisse du sommet du pic.
C = Dispersion.
D = Ligne de base.

3*a* Acide Aspartique

	A	B	C	D
A	1,000			
B	— 0,002	1,000		
C	0,913	— 0,002	1,000	
D	— 0,955	0,002	— 0,866	1,000

3*b*

	Thréonine			Sérine			
	A_1	B_1	C_1	A_2	B_2	C_2	D
A_1	1,000						
B_1	— 0,174	1,000					
C_1	0,820	— 0,004	1,000				
A_2	0,689	— 0,460	0,396	1,000			
B_2	0,467	0,185	0,523	0,183	1,000		
C_2	0,343	0,519	0,080	0,787	— 0,013	1,000	
D	— 0,890	0,326	— 0,641	— 0,881	0,340	— 0,850	1,000

cela, nous faisons généralement le graphique des résidus $(Y_i - \hat{Y}_i)$ en fonction du temps t_i (fig. 3). Si nous retrouvons sur ce graphique des tendances trop marquées, le modèle n'est pas entièrement satisfaisant; il faut donc le modifier.

Actuellement, nous envisageons d'améliorer l'ajustement des pics chromatographiques de deux manières. La première consiste à dissocier en deux gaussiennes un pic mal traduit par une seule. La seconde cherche à modifier la forme analytique de ces pics, par exemple, en remplaçant des gaussiennes par des fonctions bêta qui peuvent introduire une dissymétrie de chaque pic.

Cependant, ces nouvelles approches risquent de conduire à des conclusions très différentes de celles obtenues par la méthode utilisée jusqu'à présent. Ainsi, dans le cas d'un pic dissymétrique, les concentrations en acides aminés peuvent

varier du simple au double selon le mode de calcul : décomposition du pic en deux gaussiennes (la première correspond au premier acide aminé, la deuxième à un autre substrat) ou évaluation globale de sa surface qui sera intégralement attribué à l'acide aminé.

Conclusions

Afin d'automatiser la saisie et le traitement des renseignements obtenus à partir d'un analyseur d'acides aminés, nous avons relié par câble cet appareil à un ordinateur MITRA 15.

Les problèmes techniques posés par l'éloignement relatif (200 mètres) de deux unités, l'acquisition, la transmission et la réception des informations en temps réel sont résolus.

Ultérieurement, l'enregistrement sur cassettes pourrait remplacer si nécessaire la liaison directe.

Le traitement des données a nécessité la mise au point ou l'adaptation de méthodes d'études.

Dans un premier temps, une transformation préliminaire des renseignements « bruts » de l'analyseur en données « propres » a permis un calcul automatique des surfaces des pics chromatographiques.

Dans un deuxième temps, partant de données pour lesquelles la moyenne a été calculée sur 6 secondes, nous avons cherché à modéliser le système. Une telle démarche permet une analyse mathématique plus fine des chromatogrammes. Elle apporte un gain appréciable dans l'interprétation des résultats car elle permet de quantifier une part importante des erreurs. En outre, elle résoud des pics partiellement confondus qui ne peuvent être dissociés par la méthode manuelle.

Les résultats obtenus par les trois méthodes ont été comparés.

Nous avons noté une bonne concordance entre les méthodes manuelles et automatiques. Cependant, la méthode approfondie a fourni des valeurs de surface légèrement plus faibles. Les écarts de concentrations en acides entre les méthodes sont néanmoins très atténués par le mode de calcul faisant intervenir un coefficient de correction. Ce coefficient lié au standard interne est évidemment différent pour chacune des trois méthodes.

Actuellement, nous pensons que l'une des principales difficultés d'utilisation de la méthode approfondie réside dans la définition de la ligne base. Nous avons vu grâce aux matrices de corrélation toute l'importance de ce facteur et le rôle qu'il joue dans le résultat final. Ce doit être, avec la recherche de modèles décrivant au mieux le phénomène, un de nos axes de travail.

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Summary

Automatic analysis of chromatogramme records

Real-time acquisition and automatic processing of data from an Amino-Acid Analyser have been realized by connecting it to a Mitra 15 computer.

Tape recording replaces the direct line when real-time processing is not necessary.

Processing methods involving have been performed together with the dissociation of overlapping peaks.

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Possibilité de dosage simultané sur autoanalyseur de l'acide galacturonique et des oses neutres lors de la détermination des substances pectiques

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Résumé

L'emploi conjoint du métahydroxydiphényl)MHDP) et de l'orcinol sulfurique apparait nécessaire pour la caractérisation des substances pectiques, notamment lors de leur étude par fractionnement chromatographique : le MHDP dose l'acide galacturonique seul; l'orcinol sulfurique dose à la fois l'acide galacturonique et l'ensemble des oses neutres; il est donc possible d'individualiser ceux-ci de façon relativement simple et rapide. On décrit un montage sur analyseur à flux continu permettant de doser simultanément l'acide galacturonique et l'ensemble des oses neutres à la cadence de 15 échantillons /heure.

Mots clés : *analyse automatique, substances pectiques, acide galacturonique, oses neutres.*

Introduction

Le dosage quantitatif des substances pectiques est le plus souvent réalisé par la détermination de leur teneur en acide galacturonique; les méthodes colorimétriques sont préférées aux méthodes acidimétriques ou gravimétriques, et, le réactif carbazol est le plus généralement utilisé. Celui-ci n'est cependant pas spécifique de l'acide galacturonique et de nombreuses autres substances interviennent dans la réaction colorée; il peut être alors nécessaire d'opérer une correction pour tenir compte de ces substances interférentes, en général des oses (Spik et Montreuil, 1964).

Un nouveau réactif, le méta-hydroxydiphényl (MHDP) a été proposé (Blumenkrantz et Asbœ-Hansen, 1973) et son emploi tend à se répandre pour le dosage des substances pectiques (Ahmed el Rayah et Labavitch, 1977; Ro-

BERTSON, 1979). THIBAULT (1979) a réalisé l'automatisation de la méthode de dosage avec ce réactif et montré sa spécificité.

Les substances pectiques étant des hétéropolysaccharides renfermant des oses neutres, la seule détermination de leur teneur en acide galacturonique est parfois insuffisante et la connaissance de leur richesse en oses est nécessaire. Pour ce dosage, les réactifs les plus communément utilisés sont le phénol, l'anthrone ou l'orcinol, en milieu sulfurique (MONTREUIL et SPIK, 1963). Mais des réactifs réagissent aussi avec l'acide galacturonique; cependant, comme il est possible de connaître la teneur exacte de ce dernier composé avec la réaction du MHDP, il est facile d'en tenir compte pour obtenir le dosage précis des oses.

Ce double dosage apparaît nécessaire lors du fractionnement par chromatographie liquide des substances pectiques par exemple. L'analyse en flux continu étant une aide précieuse pour de tels travaux nous avons réalisé un montage sur autoanalyseur combinant la réaction du MHDP et celle de l'orcinol sulfurique et permettant d'effectuer simultanément les deux dosages de façon relativement rapide et sûre.

Méthode proposée

1. — *Principe*

Dans le cas des deux réactions, le principe du dosage repose sur la formation d'un complexe coloré entre les dérivés furfuryliques obtenus sous l'action de l'acide sulfurique concentré et chaud et le réactif considéré.

Le dosage de l'acide galacturonique (AGA) au moyen du MHDP est effectué selon le schéma de THIBAULT (1979) dans lequel nous avons inclus une bobine de délai pour effectuer automatiquement la saponification nécessaire (THIBAULT), 1979).

Le dosage automatique des oses par l'orcinol-sulfurique a été employé par plusieurs auteurs avec des modalités parfois un peu différentes. Le maximum d'absorption du complexe coloré oses/orcinol sulfurique se situe à 420 nm : DEMAILLE *et al.*, (1965) utilisent cette réaction à 505 nm; SZYLIT (1973) a choisi la longueur d'onde de 520 nm afin d'éliminer l'interférence des acides aminés présents dans son milieu; TOLLIER et ROBIN (1979) préfèrent utiliser la sensibilité maximum de la réaction à 420 nm.

Dans le montage que nous avons réalisé, le dosage de l'acide galacturonique et celui de l'ensemble oses + acide galacturonique sont effectués simultanément sur la même prise d'échantillon. Le matériel est ainsi réduit au minimum : un seul distributeur d'échantillons et une seule pompe proportionnante sont nécessaires. Un gain de temps appréciable, de même qu'une bonne sécurité d'analyse sont obtenus, puisque les deux dosages se déroulent à partir du même prélèvement. Connaissant, par la réaction du MHDP, la teneur en acide galacturonique de l'échantillon, il est facile d'en tenir compte dans le résultat du dosage par l'orcinol-sulfurique et d'obtenir ainsi la valeur exacte de la concentration en oses. Certains résultats de dosages sur analyseur à flux continu et utilisant l'acide sulfurique concentré sont entachés d'une erreur systématique due à la viscosité de l'acide sulfurique; nous avons montré le moyen de corriger ce « biais », correction possible sur les résultats des deux dosages lorsque cela est nécessaire (SOUTY *et al.*, 1979).

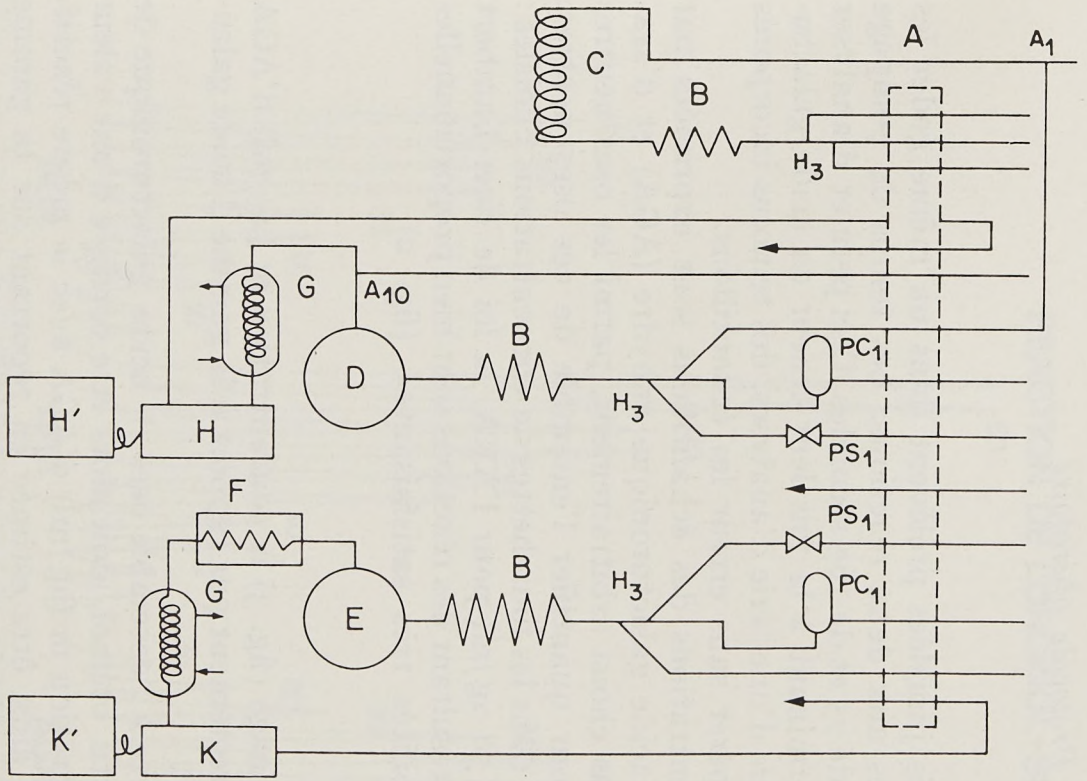

FIG. 1. — *Schéma du dispositif utilisé pour le dosage des substances pectiques simultanément par le métahydroxydiphényl (MHDP) et par l'orcinol sulfurique*

* : tubes Acidflex.
A :pompe proportionnante.
B : bobines de mélange : long : 0,16 m; ∅ int : 1,6 mm.
C : bobine de délai, long. 12 m; ∅ int. : 1,6 mm.
D : bain-marie à 80 °C avec bobine de long. : 0,24 m.
E : bain-d'huile à 115 °C avec bobine de long. : 12 m.
F : sensibilisateur de réaction UV, bobine de long. : 0,16 m.
G : réfrigérants à eau.
H et H' : spectrophotomètre réglé à 520 nm et enregistreur.
K et K' : spectrophotomètre réglé à 450 nm et enregistreur.

Foncrion tubes	débit (ml/mn)	couleur
Débullage		
NaOH N/5	0,10	O/V
Echantillon	0,60	blanc
Air	0,42	orange
Retour	*0,92	jaune
Cuve		
MHDP	0,10	O/V
Reprise		
Echantillon	0,16	O/J
H_2SO_4 pur	*1,44	vert
Air	1,20	jaune
Rinçage	2,00	vert
distributeur		
Air	0,80	rouge
Orcinol sulfurique	*0,92	jaune
Echantillon	0,10	O/V
Retour cuve	*0,64	rouge

Les annotations A_1, A_{10}, PC_1, PS_1, H_3, se réfèrent à la nomenclature Technicon. Le distributeur n'est pas représenté sur le schéma et les deux tubes d'échantillon sont couplés pour prélèvement simultané dans le même godet.

La vitesse de prélèvement est de 15 échantillons/heure : les temps de pompage sont respectivement de :
75 secondes pour l'échantillon. — 165 secondes pour le rinçage.

2. — *Appareillage*

La chaîne d'analyse utilisée (fig. 1) est constituée à partir de différents éléments. Elle comprend : un distributeur d'échantillons HOOK et TUCKER, une pompe proportionnante I TECHNICON, une bobine de délai d'environ 30 minutes, un bain marie thermostaté à 80 °C, un spectrophotomètre TURNER 330 (réglé à 520 nm) équipé d'un système de linéarisation du signal et d'enregistrement pour la réaction du MHDP; un bain d'huile thermostaté à 115 °C suivi d'un sensibilisateur UV, un spectrophotomètre TURNER 330 (réglé à 450 nm) équipé lui aussi d'un système de linéarisation du signal et d'enregistrement pour la réaction de l'orcinol.

3. — *Réactifs*

— Acide sulfurique à 98 p. 100, $d = 1{,}83$ (Prolabo).

— MHDP (Eastmankodak) : 0,04 p. 100 en solution dans la soude 0,125 N.

— Orcinol-sulfurique : 0,1 p. 100 d'orcinol monohydrate cristallisé pur (Fluka) en solution dans l'acide sulfurique à 70 p. 100.

— Acide galacturonique monohydraté, arabinose, purs (Fluka).

4. — *Protocole opératoire*

a) *Analyse*

Deux pipettes distinctes mais jumelées prélèvent dans un même godet les quantités d'échantillon nécessaires aux deux réactions. Les temps de pompage et de rinçage sont respectivement de 75 et de 165 secondes. Ceci permet d'analyser 15 échantillons à l'heure, en déterminant à la fois leur teneur en acide galacturonique et en oses neutres. Au cours d'une série d'analyses, des témoins incorporés judicieusement permettent de repérer sans erreur les échantillons.

Dans tous les cas, les concentrations des échantillons sont exprimées par rapport à des gammes étalons d'acide galacturonique anhydre (AGA) et d'arabinose (ARA), ose que nous avons choisi arbitrairement, parmi les oses neutres liés aux substances pectiques, pour quantifier l'ensemble de ces oses neutres. Dans nos conditions opératoires et dans les fourchettes de concentrations choisies : 0 à 60 µg/ml pour l'AGA et 0 à 50 µg/ml pour l'ARA, la loi de Beer-Lambert est respectée, les hauteurs des pics résultant des réactions sont bien proportionnelles aux concentrations et les sensibilités très satisfaisantes. (fig. 2)

b) *Calcul*

A partir des gammes d'étalonnage (fig. 3) la concentration m (µg/ml) d'AGA dans l'échantillon est exprimée directement par rapport à la gamme d'acide galacturonique avec le réactif MHDP.

La densité optique (A) relative à l'ensemble oses + acide galacturonique de l'échantillon, obtenue avec le réactif orcinol, doit alors être corrigée d'une valeur égale à celle que donne la concentration m (µg/ml) d'AGA avec le même réactif. La concentration n en oses peut ainsi être estimée en reportant sur la gamme d'étalonnage d'arabinose la D.O. corrigée ($P = A - B$) relative aux seuls oses de l'échantillon.

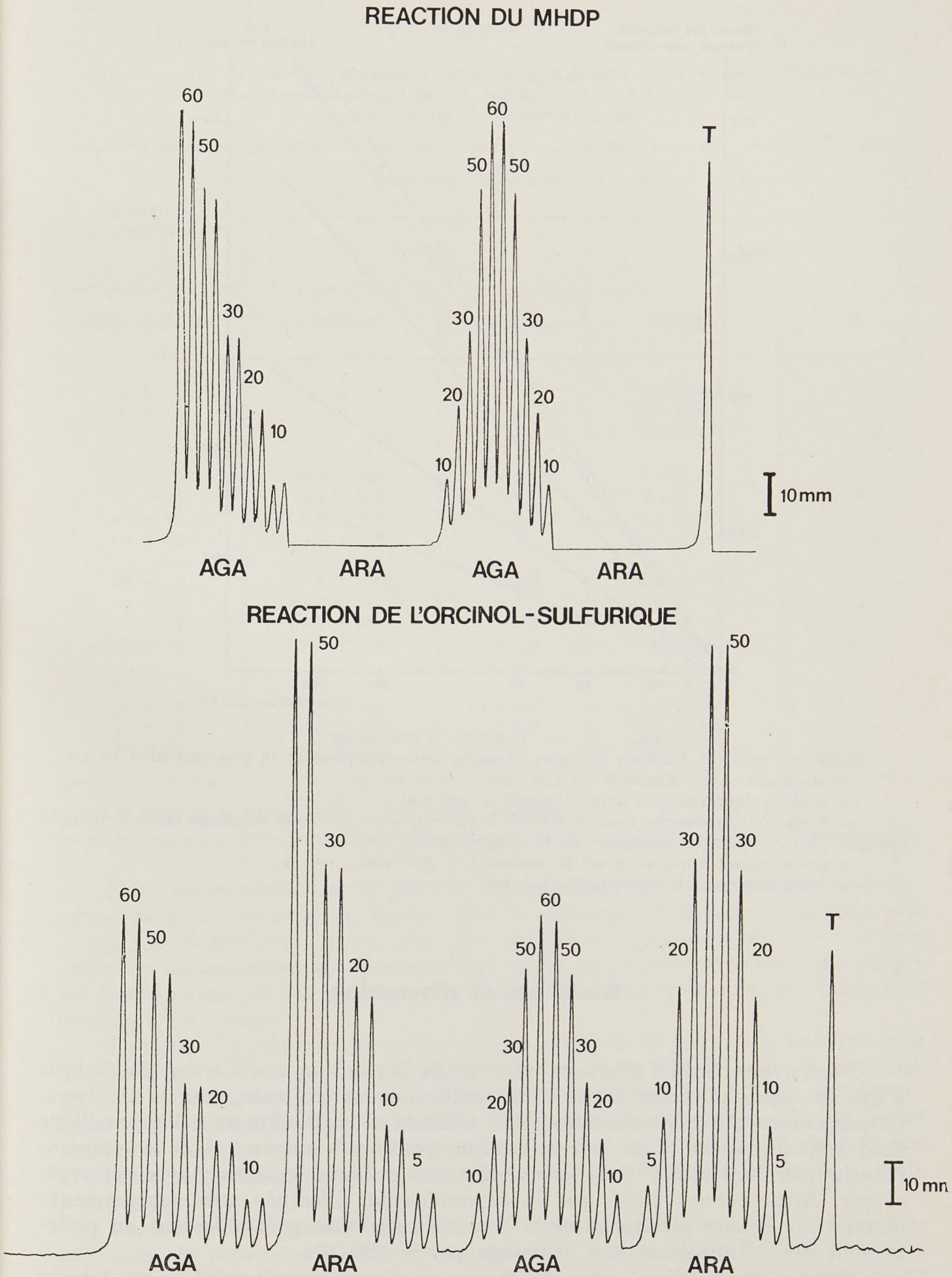

FIG. 2. — *Exemple de résultat obtenu pour des gammes d'étalonnage d'acide galacturonique (AGA) et d'arabinose (ARA) avec les réactions du MHDP et de l'orcinol sulfurique*
(T = témoin servant de point de repère).

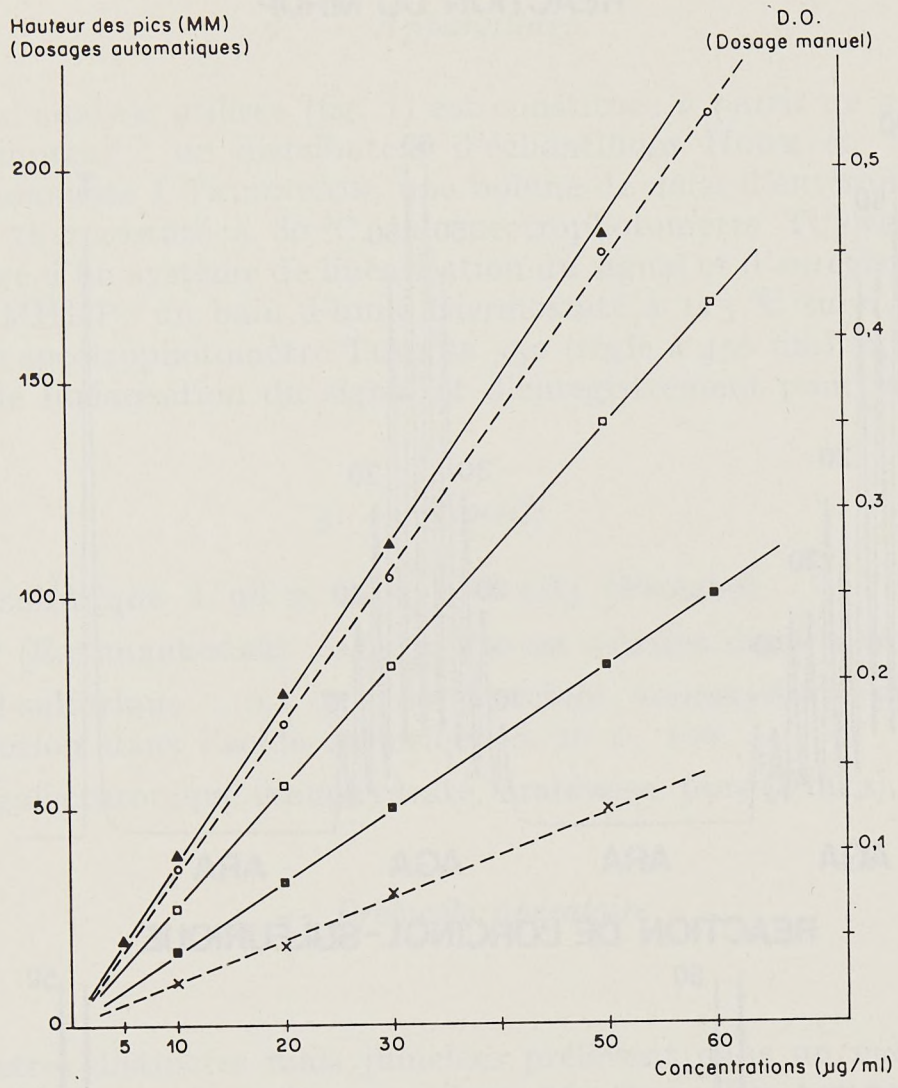

FIG. 3. — *Courbes d'étalonnage*
Relations entre la hauteur des pics (dosages automatiques) et la concentration en :
— arabinose avec l'orcinol à 450 nm — — △ — —
— acide galuctorunique avec l'orcinol à 450 nm —— ■ ——
— acide galacturonique avec le MHDP à 520 nm —— □ —— et relations entre la densité optique (D.O.) (dosages manuels) et la concentration en :
— acide galacturonique avec le carbazol à 520 nm - - - - ● - - - -
— arabinose avec le carbazol à 520 nm - - - - × - - - -

Résultats et discussion

Nous avons choisi d'effectuer la lecture de la réaction avec l'orcinol sulfurique à 450 nm, après plusieurs essais à différentes longueurs d'onde, pour le bon compromis obtenu entre la sensibilité de la réaction et sa fidélité avec l'appareillage dont nous disposons. C'est la même raison qui nous a amené à fixer la température du bain-marie à 115 °C, la sensibilité de la réaction augmentant avec la température. Nous avons vérifié que la saponification effectuée automatiquement donnait les mêmes résultats que si elle était effectuée préalablement au prélèvement des échantillons pour le dosage par le MHDP.

Nous avons appliqué cette technique de dosage simultané à des solutions synthétiques de concentrations connues en acide galacturonique et en arabinose en mélange (tabl. 1).

TABLEAU I

Recouvrement de quantités connues d'un mélange d'acide galacturonique et d'arabinose dosées sumiltanément par le MHDP et l'orcinol sulfurique
(moyennes de 8 déterminations)

Quantités théoriques	Quantités recouvrées par le dosage simultané				
	AGA		+	ARA	
AGA + ARA	Dosage	Δ		Dosage	Δ
0 + 5	0		+	5,8 ± 0,1	+ 4
0 + 15	0		+	15,0 ± 0,2	0
0 + 25	0		+	25,3 ± 0,2	+ 1,2
10 + 0	9,5 ± 0,2	— 5	+	0,4 ± 0,2	
10 + 5	9,8 ± 0,1	— 2	+	5,1 ± 0,07	+ 2
10 + 15	9,8 ± 0,1	— 2	+	15,0 ± 0,2	0
10 + 25	9,6 ± 0,1	— 4	+	24,7 ± 0,2	— 1,2
20 + 0	20,1 ± 0,09	+ 0,5	+	0,2 ± 0,1	
20 + 5	20,2 ± 0,2	+ 1	+	4,7 ± 0,09	— 6
20 + 15	20,1 ± 0,2	+ 0,5	+	14,1 ± 0,2	— 6
20 + 25	20,1 ± 0,1	+ 0,5	+	0,2 ± 0,1	— 6
30 + 0	30,0 ± 0,2	0	+	0,1 ± 0,06	
30 + 5	30,1 ± 0,2	+ 0,3	+	4,5 ± 0,1	— 10
30 + 15	30,2 ± 0,3	+ 0,7	+	13,4 ± 0,3	— 11
30 + 25	30,0 ± 0,2	0	+	22,6 ± 0,2	— 10

Δ = Écart en p. 100 par rapport à la valeur théorique.

Nous avons vérifié que la précision des méthodes est identique à celle déterminée pour la méthode de dosage, par le carbazol, c'est-à-dire meilleure que ± 3p. 100 (Souty *et al.*, 1979). Dans ces conditions, nous pouvons dire que nous obtenons un recouvrement pratiquement exact de l'acide galacturonique et que les écarts constatés par rapport à la valeur théorique sont dans les limites de confiance du dosage.

Le taux de recouvrement de l'arabinose apparaît un peu moins bon que celui de l'acide galacturonique puisque nous constatons un déficit pouvant aller jusqu'à 10 p. 100 lorsque la concentration de l'échantillon en AGA est très forte : la proportion respective de l'AGA et de l'ARA dans les échantillons de substances pectiques étant en général très différente et en faveur de l'AGA, il s'ensuit que l'erreur absolue réalisée sur le dosage des oses neutres n'est pas excessive et reste acceptable.

Le tableau 2 rapporte les résultats d'analyses effectuées sur 7 pectines commerciales de provenances diverses et que nous avons dosées également par la méthode manuelle au carbazol (Dietz et Rouse, 1953). Le MHDP donne toujours une valeur plus faible que le carbazol ce qui prouve bien, comme l'a souligné Thibault (1979), sa plus grande spécificité.

TABLEAU 2

Résultats de la caractérisation de différentes pectines en utilisant le dosage simultané par le MHDP et l'orcinol sulfurique

Pectines	Teneur en méthanol % (1)	Dosage au Carbazol (méthode manuelle) (2)		Dosage simultané OSES + AGA selon la méthode proposée		
		AGA %	D.E (3)	ARA %	AGA %	D.E. (3)
1	7,6	89,7	51	7,5	79,5	58
2	2,8	92,9	18	10,6	77,1	22
3	6,5	88,8	44	8,2	75,2	52
4	6,5	67,6	58	17,7	57,3	68
5	3,0	70,6	26	16,3	55,3	33
6	7,6	76,7	60	16,0	65,9	70
7	8,5	86,6	59	9,3	79,9	64

(1) Méthode de Wood et Siddiqui (1971).
(2) Méthode de Dietz et Rouse (1953).
(3) D.E. = degré d'estérification = $\frac{\text{nombre de moles de méthanol}}{\text{nombre de modes d'ac. galact.}} \times 100$.

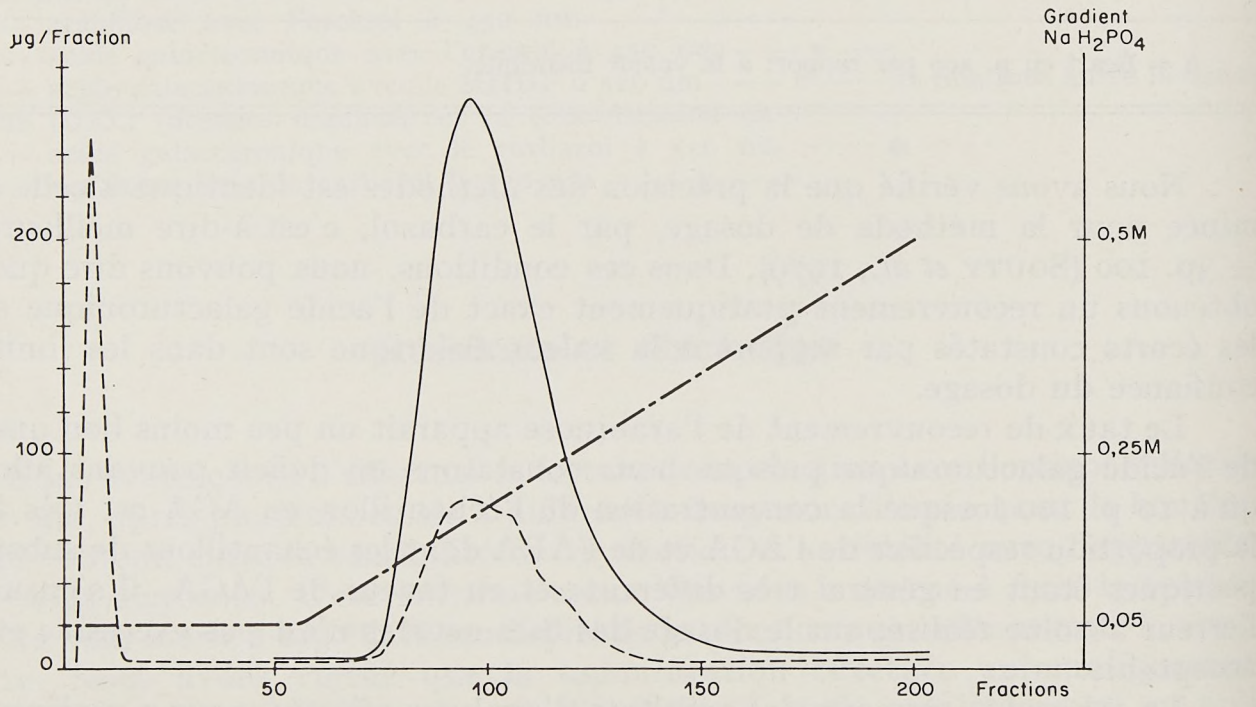

Fig. 4. — *Exemple de fractionnement de la partie soluble à l'eau des substances pectiques de l'abricot. Les dosages simultanés par le MHDP et par l'orcinol sulfurique de l'effluent de la colonne permettent de distinguer la partie oses neutres (courbe - - - -) de la partie acide galacturonique* (courbe ————).
—— - —— gradient tampon phosphate.

Mais l'intérêt principal de l'utilisation de cette technique est son application à l'analyse des effluents d'une colonne chromatographique. La séparation en chromatographie liquide par échange d'ions ou par filtration sur gel, semble être un moyen d'approfondir la connaissance de la structure chimique des pectines (Neukom *et al.*, 1960; Van Deventer-Schriemer et Pilnik, 1976; Masuda *et al.*, 1979) et c'est dans ce but que nous utilisons plus spécialement ces dosages. Un exemple de chromatographie réalisée est donné figure 4 et illustre l'intérêt du double dosage permettant d'obtenir une caractérisation plus complète des fractions pectiques. Ce type d'analyse fait l'objet d'un travail en cours et les premiers résultats montrent la possibilité de mieux connaître ces substances et peut être d'expliquer certains caractères de l'évolution des fruits au cours de leur maturation ou des traitements technologiques.

Conclusion

La simultanéité des dosages de l'acide galacturonique par le MHDP et de celui de l'ensemble oses + acide galacturonique par l'orcinol sulfurique, appliqués à des échantillons de substances pectiques, présente des avantages certains de rapidité et de sureté pour leur étude fine et permet d'envisager de nouveaux développements dans la connaissance de ces substances et de leurs implications technologiques.

Reçu pour publication en juin 1980.

Summary

Simultaneous determination with an automated analyser of galacturonic acid and neutral sugars in pectic substances analysis.

The use of both chromogens : methaydroxydiphenyl (MHDP) and sulfuric orcinol, are necessary to characterize pectic substances, in particular when liquid chromatographic techniques are involved: MHDP determines only galacturonic acid while orcinol gives in the same time galacturonic acid and neutral sugars. It is then possible to have the true rate of galacturonic acid and neutral sugars in the sample. The simultaneous use of the two reactions on automated analyser is developped which allows the determination of 15 samples per hour.

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Workshop on the physico-chemical properties of wheat gluten proteins

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Workshop on the physico-chemical properties of wheat gluten proteins

NANTES, FRANCE, APRIL 28TH to 30TH 1980

SPONSORED BY THE « INSTITUT NATIONAL DE LA RECHERCHE AGRONOMIQUE »

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RÉSUMÉ DES SESSIONS

Session I

Extraction and composition of Wheat proteins

R. LASZTITY and J. MOSSÉ

(Chairmen)

It is a hard work to discuss extraction of protein, because of the very empirical feature of this topic. Indeed, many positive new steps have been done thanks to the use either of solvents or of methods the efficiency of which is not always easy to explain with scientific and unquestionable arguments.

There are several reasons behind the difficulties of the problem of extraction.

1) The almost *anhydrous* state of wheat grain, the development of which arises from possible agregation, partially due to dessication. Any knowledge of grain protein composition, structure and properties requires the extraction of these proteins and to dissolve them using solvents and conditions as mild as possible, without drastic conditions.

2) The *strong chemical heterogeneity* of grain, which contains so many biochemical components : lipids, glucids, enzymes, nucleic acids, polyphenols, small molecules of very differents kinds and so on.

From this point of view, the way of working with isolated protein bodies, as it is the case in the work reported by Dr MIFLIN is very promising.

3) The *biological heterogeneity* of grain tissues, with the presence of at least two distinct individues, embryo and endosperm, with their different gene doses.

4) The relative *small size of wheat grain :* with its weight of 30 to 50 mg, it is a rather minute material for the biochemist.

Nevertheless, several laboratories have succeeded in carrying out electrophoreses with single grains as it was mentioned several times during this meeting. Electrophoresis, in its many forms, remains both the most powerful and efficient method, and also the most rapid and simple technique able to follow the successive steps of any extraction procedure.

5) There is unfortunately a frequent *confusion between solubility and extractibility* which does not correspond to the same characteristics. Because of the

multiplicity of components, it is possible to extract a pool of seed proteins with a solvent mixture in which they may not be soluble or vice versa.

6) *Extraction* may be carried out either for *biochemical and physiological studies*, or for *industrial* purposes but involved processes will probably not be the same.

7) *Extraction* can be performed either in order to study a *limited group of proteins*, or in order to put them all in solution by an *exhaustive procedure* (if there is one). « Total » extraction are very seldom exhaustive and one can wonder wether unextracted proteins are really similar to the already extracted ones. It could be that a few fractions having a very significant function still remain unextracted. We can notice that Dr KOBREHEL, by the use of fatty acid salts, reaches an extraction amount of around 90-95 p. 100.

But even when extracting a large amount of grain proteins, and even when using powerful and sophisticated electrophoretic techniques, the lack of exhaustivity and the limited informations arising from electrophoresis are often forgotten. May we remind that roughly two thirds of isoenzymes or isoproteins are not separated by electrophoresis.

In other words, anydrous state and small size of wheat grain, chemical and biological heterogeneity, differences between solubility and extractivity are many point which make hard to the study of wheat proteins.

Concerning the solvents frequently used, it can be seen that several of them are just the classical solvents able to dissociate quaternary structure of proteins (if present) : such as in the case of detergents or soaps, (sometimes called amphipathic substances because one arm of their molecule is non polar and the other one polar) or such as with grain lipids mentioned by Dr MIFLIN or as urea and also as cetyltrimethylammonium bromide.

We can now try to sumarize very briefly the main conclusions of the 3 speakers of the first session.

Dr GRAVELAND has pointed out the advantages of a combined extraction separation using ultracentrifugation, and subsequent extraction. Based on the investigation of the properties of SDS-insoluble proteins a new hypothesis was elaborated for the mechanism of redox reactions included in the process of cleavage of S-S-bonds.

The solubilising effect of different fatty acid salts and the kinetics of different protein fraction solubilization have been discussed by Dr KOBREHEL. The differences in the solubility of different protein fractions give a possibility to predict to baking value of wheats.

Various solvents have been investigated by Dr MIFLIN and his coworkers in order to isolate exhaustively the protein components of wheat flour. It has been emphasized that a well planned combined extraction is a necessary tool in obtaining well defined fractions. One of the main results is that extracted proteins contain the same prolamins when their extraction procedure is applied as well to whole wheat flour as to washed gluten or also to preliminary isolated protein bodies.

Concerning the solvents, it can be noticed that six susbtances (or groups of substances) are classically used, more or less combined one with another.

— Alcohols, mainly ethanol and propanol.
— Detergents, and mainly SDS.
— Fatty acid salts, the use of which is perhaps the most recent.

— Reducing agents, like 2-mercaptoethanol or dithiothreitol.
— Weak acids, like acetic or lactic acids (or sometimes high pH near 8 or 9).
— Urea, one of the best solvents for protein because of its powerfull disrupting effect upon hydrogen bonds.

Now, for the next future, there is no really new way to propose. But efficiciency of research could be improved by several means, like following ones:

1) Working as often as possible, at least partially on the same varieties and preferentially on the same samples of the same varieties.

2) Using different solvents and extraction procedure but comparing them with the same methods and in the same laboratories.

3) Using just the same extraction procedure but comparing the extracted proteins with different techniques in different laboratories.

4) Continuing both to extract and fractionate pure polypeptide chains or real subunits and on another side, trying to clone copy DNAs coding for the storage proteins in order to eliminate a too large polymorphism and to be able to study a single molecular species.

5) Completing the work of comparison on the storage proteins which are inside of protein bodies with these ones which are outside of them.

Indeed, and this will be our conclusion, several questions remain without answer:

— Do glutelins exist with a very elaborated quaternary structure or does the agregated state arise partially from extraction and mixing processes?
— What are the proteins included inside of protein bodies: do these organelles contain the whole prolamins? This is quite possible. Do they contain significant amount of other proteins like a part of glutelins? This is likely.
— Are there glutelin polypeptide chains which are both located inside and outside the protein bodies? If there are, do they always have precursors with signal peptides?
— Do the reducing agents only release the real protein subunits during extraction or do they possess any other function? Perhaps any experiment of milling and extracting under nitrogen gaz without any oxygen could contribute to bring significant informations. In any case, the redox reactions occurring in the presence of air and their effect on the disulfide — thiol system of wheat proteins need further investigations.

We have to stop here the beginning of such a question list. We can hopefully expect many new advances in the very next future. Probably one of the most efficient means is the friendly climate like that everybody has felt during this workshop.

Session II

Wheat gliadins: structure and properties

D. D. KASARDA and B. J. MIFLIN

(Chairmen)

I. The theme of the session was to classify the gliadin proteins on the basis of the following criteria:

(1) Starch — or lactate polyacrylamide — gel electrophoresis. This method divides the gliadins up into α, β, γ and ω sub-groups based upon their mobility.
(2) Amino acid composition.
(3) N-terminal amino acid sequence.
(4) Hydrophobicity and other physico-chemical properties.

II. The significant results presented included the observations that:

(1) The α, β and γ subgroups have similar amino acid compositions whereas the ω's are clearly different in that they contain little or no S-amino acids and higher contents of glutamate and proline.
(2) On the basis of N terminal sequences so far determined the gliadins fall into three different types. One sequence is similar for the α, β and γ_1 gliadins, one for γ_2 and γ_3 gliadin, and one for ω-gliadin. The ω gliadin sequence is similar to the N-terminal sequence of a prolamin from another species.
(3) The conformational structure of the gliadins (particularly the α gliadins) is related to their visco-elastic properties.
(4) Classification of the proteins on the basis of surface hydrophobicity, using hydrophobic interaction chromatography, does not correspond to the categories defined by gel electrophoresis.

III. Future topics for research, suggested by the session, were:

(1) The need to define the location and organisation of the structural genes for the different groups and to relate these different genes to the ancestors of cultivated wheat.
(2) To determine the importance of gene duplication in leading to the presence of polymorphic series of gliadins, particularly by determining the sequences of nucleic and amino acids in the genes and proteins respectively.
(3) To determine the physico-chemical nature of the component gliadin polypeptides in relation to their tertiary structure and properties of the surface of the proteins as it affects their interaction with other molecules.
(4) To correlate physico-chemical classifications of the gliadin proteins to genetic and electrophoretic classes.

Session III

Wheat gliadins: nomenclature and varietal identification

J. C. AUTRAN and W. BUSHUK

(Chairmen)

During this third session devoted to wheat gliadins with regard to protein nomenclature and varietal identification, we had three most interesting papers, that really made excellent contributions in this field, from:

— Dr R. TKACHUK, Canada.
— Dr A. RYBALKA, S.S.S.R.
— Dr J. L. BATEY, Australia.

First, Dr TKACHUK presented useful modifications of W. BUSHUK' polyacrylamide gel electrophoresis procedure in view to improve cultivar identification system. These modifications concerned mainly the introduction of a high voltage that allows to reduce electrophoresis time. On the other hand, the use of a vertical apparatus make possible to cast at the same time several thin gel slabs that are also easier to load. With a such system it is therefore possible to run much more samples in a shorter time but some special cautions should be taken since high voltages can generate more heat.

Second, Dr RYBALKA's talk covered both genetic basis of hordein nomenclature and Pr SOZINOV's talk on wheat breeding based on gliadin components. These results consisted of a new approach of prolamin nomenclature based on the search of the products of gene blocks. They evidenced the linkage between prolamine blocks and other characteristics like disease resistance or quality data. It was shown that breeding systems using prolamin electrophoregrams can be now developed and that investigations for such linkages are more likely to succeed if based on blocks and not on single bands. This system is however more helpful in breeding and in genetics studies than in varietal identification since it is more sophisticated and requires a tremendous amount of work including aneuploid studies, crossings and analyses of thousands of kernels from the offsprings.

Third, Dr BATEY's paper consisted of two important results:

— a further modification of the P.A.G.E. system by using gradient concentration gels which gives some advantages in resolution upon starch or uniform P.A.G.E., allows to differenciate more varieties and is available using preformed gels avoiding acrylamide monomer toxicity.

— computer applications for handling or processing different electrophoretic data, such as in varietal identification, in building of genetic trees or taxonomic keys, in calculating how closely are different genotypes or species.

Concerning *recommendations for future progress*, it must be recalled that I.C.C. has now a working group on varietal identification through electrophoretic techniques (Chairman : Pr. W. BUSHUK) which can be joined by everybody is interested in this field.

We know of course that everybody in each country has already developed his own technique: polyacrylamide slabs in Canada, starch slabs in France, vertical starch tubes in S.S.S.R., gradient concentration P.A.G.E. in Australia, ... Accordingly, we do not like to recommend a given technique because we cannot say that one is better than one another. It is just a matter of personal preference and experience and also of equipments that cost money. We would like that people come to some agreement on a *reference system* (and not on a routine system) just in order that those who do and publish research can speak the same language and know which band they are referring to. It is a tentative recommendation for interlaboratory agreement and for developing some day a catalog of international varieties.

As far as experimental technique is concerned, in addition to what Dr TKACHUK reported on reduction of electrophoresis time and increasing of the daily number of run samples, many other things are still to be improved including a better utilization of computer tool, for example image analysis, in varietal identification and in any kind of genetic studies.

Since we need more informations on inheritance of components and identification of quality markers in genetic pools, we should more and more develop breeding programs taking advantage of the linkage between gliadin (and also H.M.W. glutenin subunits in SDS systems) and any quality or desease resistance data.

We must not stay where we are and we all must continue to improve our techniques and, more especially under the sponsorship of I.C.C., to publich as soon as possible any new information available.

Session IV

Wheat glutelins

Prof. J. KACZKOWSKI, Dr R. TKACHUK

(Chairmen

Thème :

Conference theme apparently was to present progress and advances in the extremely difficult field of research on wheat glutenin.

Definition :

Gliadins : Proteins of wheat endosperm soluble in alcohol such as 70 p. 100 EtOH (v/v) at room temperature, and which migrate in polyacrylamide and starch gels without reduction as reasonably discrete bands, and which are not excluded during gel filtration on Sephadex G-100.

Glutenins : Proteins usually not soluble in room temperature 70 p. 100 ethanol (v/v), and which are excluded during gel filtration on Sephadex G-100 using acidic ionic detergents.

A minority thought that the above definition was artificial.

Significant results presented and discussed :

1. A possible relationship exists between some high MW wheat endosperm polypeptides and bread quality.

2. Some of the subunits have been genetically assigned to various chromosomes.

3. Some of the high MW proteins may originate from a specific cell site but a majority expressed doubt as to this hypothesis.

4. A number of people considered that the MW of many of the storage proteins is lower than previously thought. However, actual data were not presented.

5. Correlations are present between gel amounts and baking quality of most French wheats.

Future research required :

1. Develop closer relationships between physical properties of subunits and their genetic origines.
2. Define more clearly the bonds which link the various subunits together.
3. Provide more quantitative data as to subunit amounts.
4. Sub-cellular location of the various subunits should be established.
5. Publications should have illustrated SDS-PAGE analysis of total flour proteins plus the fraction studied.
6. Continue to clarify relationships between molecular structure and gluten components.
7. Continue to clarify what the relative importance of covalent and non-covalent interactions is to the physical properties of gluten.
8. Research should continue to the genetic possibility of extending the variability within bread wheats by incorporating genes from other species.

Session V

Gluten

J. A. Bietz and V. G. RYADCHIKOV

(Chairmen)

Papers presented in this session were concerned with the relation between gluten's chemical structure and properties, with the chemical modification of gluten's structure, and with the interaction of gluten with other flour constituents.

The most significant results reported in the session were as follows. (1) Four types of disulfide bonds differing in accessibility and reactivity were proposed. Their distribution varies between wheats, and may be related to quality. Amide groups and hydrophobic character also seem to be related to quality. (2) Specific chemical reactions indicated that the exterior of gluten molecular complexes is hydrophilic, and their interiors are hydrophobic. Alkali causes rapid loss of gluten's functional properties through loss of cystine; gliadin was more susceptible to functional change than was glutenin. (3) Lipid-gluten interactions significantly affect dough properties. Models were presented showing how lipid addition, particularly glycolipids, can have an improving action in dough and

bread. (4) When gluten is dried above 60°, loaf volume, gluten extensibility, and glutenin solubility decrease significantly through unfolding followed by formation of additional intermolecular disulfide bonds. (5) Gluten was shown to be very sensitive to modification; it is easy to degrade, but difficult to improve. (6) The contribution to wheat quality of interactions of gluten with non-protein flour constituents was demonstrated, and new methodology revealed the great importance of non-covalent bonding in gluten's structure.

Future research needs which were discussed could be grouped into four distinct areas. (1) *Gluten Production.* Are optimal processes now being used? Industry wants feedback on ways to modify processing to improve baking quality. There is a possible need for standard specifications for protein content in gluten, as well as a need for wheats specifically bred for washing out gluten. (2) *Quality Tests.* Methods used to determine quality should be compared, optimized, and standardized to determine best processing conditions. Tests should be less emperical, and should relate to accurate physical measurements. New techniques, such as ^{13}C-NMR, should find use in gluten analysis, and there is a need for more non-destructive quality tests and tests for disulfide and sulfhydryl bonds. (3) *Utilization.* Good bread can sometimes be baked from " poor-quality " gluten, suggesting that different glutens have different optimum uses, and that more than one gluten quality type may be desirable. Also needed is information on how to best utilize gluten of a given variety, but differing in quality due to environmental conditions or crop year. More information on enzymic improvement of gluten is needed. It may be desirable to optimize flour's physical properties by adjusting relative amounts of gliadin and glutenin, rather than by addition of whole gluten. (4) *Structure.* Although most models for gluten's structure are seen to be converging, there is further need for determining the contributions of components such as water-soluble protein, lipids, carbohydrates, and pentosans. Relatively little is known concerning the importance of electrostatic interactions, or of interactions with water. There is a need to isolate and characterize single protein species still retaining functional properties. How does the percentage of protein in gluten relate to its properties, and what is the protein structure in long-mixing varieties? It is necessary to determine reasons for poor quality to prevent its occurence.

It was concluded that standard nomenclature for gluten is desired, and also standard terminology for disulfide bond location and reactivity. Continued multi-disciplinary efforts involving wheat breeders, chemists, processors, and users are desirable to further improve gluten. Conferences such as the Workshop on the Physicochemical Properties of Wheat Gluten Proteins are very important for interchange of information between these groups.

SESSION I

Extraction et composition des protéines de blé

Ann. Technol. agric., 1980, **29** (2), 113-123.

Extraction of wheat proteins with sodium dodecyl sulphate

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Summary

With the aid of a 1.5 p. 100 sodium dodecyl sulphate (SDS) solution, 65-70 p. 100 of the total nitrogen of wheat flour can be dissolved. The solution can be fractionated into glutelins, pentosans, albumins, globulins, gliadins and glycoproteins by successive addition of alcohol and $(NH_4)_2SO_4$.

The insoluble material in the flour residue is separated by ultracentrifugation into a bottom-layer of starch, a top-layer of gelatinous material, and a light brown layer in between (*J. Sci. Fd. Agric.*, **30** (1979), 71-84). If this light brown layer is suspended in SDS solution and centrifuged, it renders a white and a brown layer both containing high percentages of pentoses, while the brown layer moreover contains 10 p. 100 of proteinaceous material, called glycoproteins I. The top layer, or gel layer, can be seperated in a similar way into " SDS gel proteins or glutenins " and a soluble fraction called glycoproteins II.

In this paper the physical and chemical properties of the SDS-insoluble fractions and the SDS-soluble glutelins are discussed. The SDS gel proteins or glutenins are important in breadmaking: during dough-mixing they are broken down to SDS-soluble products through cleavage of their SS bonds, and after dough-mixing a re-formation of SDS gel proteins takes place. The cleavage of SS bonds is caused by electrons originating from superoxide anions (O_2^-.) and transferred by an Mn/Cu-protein complex. An Mn^{3+}-protein complex is involved in the re-formation of SS bonds from SH groups by oxidation (*Biochem. Biophys. Res.* Comm. in press).

Introduction

The proteins of wheat flour are primarily responsible for the unique viscoelastic properties of dough. It is generally assumed that differences in protein quality between wheat varieties and the action of oxidative improvers during mixing are related to fundamental differences in the gluten protein structure. Many investigators have studied the wheat flour proteins in order to acquire more information on the molecular organisation of gluten proteins. The structure of wheat proteins and especially the interactions of the proteins in the gluten

matrix have been studied, and the influence of glutenin and gliadin in determining bread-making quality has been demonstrated by rheological, baking, and protein fractionation studies.

The term glutenin was introduced by OSBORNE (1907) to describe those proteins of the wheat endosperm that were insoluble in water, salt solution or aqueous ethanol but soluble in dilute acids and alkalis. It comprises approximately 40 p. 100 of the proteins of bread wheat flour. It is the protein that contributes elasticity to a dough. Various workers have analysed this fraction further by modern biochemical methods during the past fifteen years.

It is difficult to obtain a clear picture of the term " glutenin " because it has been applied to preparations obtained in many different ways, and the term means different things to different investigators. To obtain more insight into the behaviour and composition of the gluten it is necessary to compare the results concerning the following points: starting material, extraction procedure, fractionation procedure, chemical and physical properties and their functionality (the mixing and baking characteristics).

In our investigations of wheat flour proteins we adhered to three principles: *a*) during extraction and fractionation no cleaving of disulphide bonds must take place, *b*) fractions obtained by fractionation must be of uniform composition, *c*) characterization of the fractions must be done by more than one technique.

With these principles in mind and based on the results obtained by DANNO *et al.* (1974), we have chosen an extraction with a 1.5 p. 100 sodium dodecyl sulphate (SDS) solution. No cleaving of SS-bonds takes place and a good separation between SDS-soluble and SDS-insoluble proteinaceous material can be obtained. Moreover, the SDS-insoluble material can be separated into three fractions of uniform composition. The first results of these extraction and fractionation procedures have been published last year, in (GRAVELAND et *al.*, 1979). Meanwhile, we have modified the fractionation procedure and obtained purer fractions.

Results and discussion

Extraction and fractionation procedure of wheat flour proteins

Defatted flour (5.0 g) was gently suspended in 90 ml 1.5 p. 100 SDS solution and magnetically stirred for 10 mn at 10 °C. The suspension was centrifuged at 80.000 g for 30 mn at 15 °C. Three distinct layers were formed at the bottom of the centrifuge tube (Fig. 1): a bottom layer of starch, a top layer of gelatinous material, and a light brown layer in between. The SDS-insoluble material constituted about 40 p. 100 of the total wheat flour proteins.

The SDS supernatant, containing 60 p. 100 of the total wheat flour proteins, was fractionated by addition of ethanol to 70 p. 100. After standing for 15 mn at room temperature, the mixture was centrifuged at 2.000 g for 5 mn. The precipitate was suspended in 100 ml H_2O by using a polytron homogeniser for 15 s. Centrifugation of the suspension at 20,000 g for 15 mn gave a clear supernatant (water soluble pentosans and glycoproteins IV) and a yellow precipitate (glutelins).

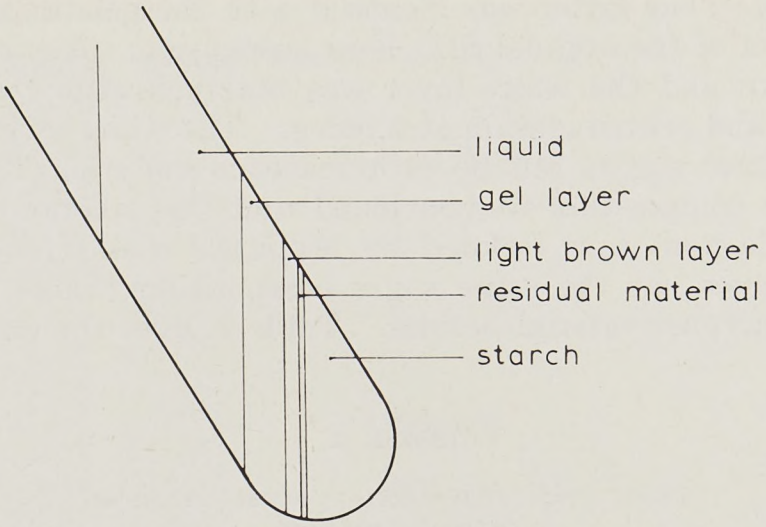

FIG. 1. — *Centrifugation of SDS-flour suspension.*

The supernatant liquid that remained after the addition of alcohol was further fractionated by adding $(NH_4)_2SO_4$ to a 10 p. 100 concentration. The precipitate contained gliadins and glycoproteins, and the supernatant contained albumins, glycoproteins and pentosans.

Characterization

SDS-insoluble light brown layer (glycoproteins I and pentosans or hemicellulose)

After resuspending the light brown layer in SDS solution using a Polytron homogeniser for 15 s and centrifugation at 30,000 g four layers were formed, from the bottom to the top a starch layer, a dark brown layer, a white layer and

TABLE I

Sugar composition of the glycoprotein fractions

Fraction	Protein content (%)	Neutral sugar content (%)	Not identified (%)	Neutral sugar composition				
				Ara (%)	Xyl (%)	Man (%)	Gal (%)	Gluc (%)
SDS-insoluble material:								
Brown layer (Glycoproteins I)	10	82	8	31	50	7	2	10
White layer (Pentosans)	0	92	8	31	54	5	1	9
Glycoproteins II . .	9	86	5	8	3	54	17	38
SDS-soluble material:								
Glycoproteins IV . .	15	79	6	30	50	2	1	17
Glycoproteins V . .	5	87	8	2	4	2	15	79

a thin layer of gel. The latter was identical with the gelatinous layer obtained after centrifugation of the original SDS flour suspension. A complete separation between the brown and the white layer was obtained after resuspending them together in water and centrifugation at 2,000 g. The white layer contains a high percentage of neutral sugars but no proteinaceous material (Table 1). On the basis of the sugar composition we concluded that this fraction is identical with the water-insoluble pentosans isolated by MEDCALF *et al.* (1968).

The brown layer has the same sugar composition (Table 1), but contains 10 p. 100 proteinaceous material besides. Table 2 gives the amino acid compo-

TABLE 2

Amino acid composition of protein fractions
(g/100 g protein)

Amino acid	Wheat				Barley
	Gel proteins or glutenins	Gliadins + glyco-proteins V	Glyco-proteins I	Glutelins	Glutelins
Lysine	1.53	0.53	4.34	5.52	5.10
Histidine	1.62	1.94	2.61	2.87	2.71
Arginine	3.08	2.13	6.30	7.15	5.56
Aspartic acid + asparagine	2.48	2.32	8.46	6.94	6.37
Threonine	2.42	1.79	4.32	3.91	3.90
Serine	4.48	4.56	5.31	4.89	5.00
Glutamic acid + glutamine	30.46	44.93	11.07	15.24	18.92
Proline	8.02	15.30	5.49	5.12	7.21
Glycine	3.35	1.47	7.47	4.19	4.12
Alanine	2.05	1.76	5.31	4.96	4.62
Valine	3.25	3.82	5.31	5.97	5.93
Méthionine	1.46	1.29	0.31	2.36	2.21
Isoleucine	2.84	4.14	3.60	4.40	4.21
Leucine	5.56	6.65	6.75	8.08	7.75
Tyrosine	3.49	2.66	4.05	4.30	3.95
Phénylalanine	3.45	6.52	4.32	5.25	5.21
Cystine ½	1.47	2.01	3.56	2.03	1.86

sition of this proteinaceous material. It is chemically bonded to the sugar complex, we therefore classify it as glycoproteins I. The sugar compositions of the white and brown layers differ somewhat from the results presented in the above-mentioned publication. The difference must be attributed to impurities, especially starch, in the light brown layer.

Gel layer (SDS-gel proteins or SDS-insoluble glutenins and glycoproteins II)

The gel layer was separated into two fractions as follows: it was suspended in ethanol, magnetically stirred for 10 mn at 5 °C, and centrifuged at 5,000 g. The residue consisted of polymers with pronounced elastic properties and contained

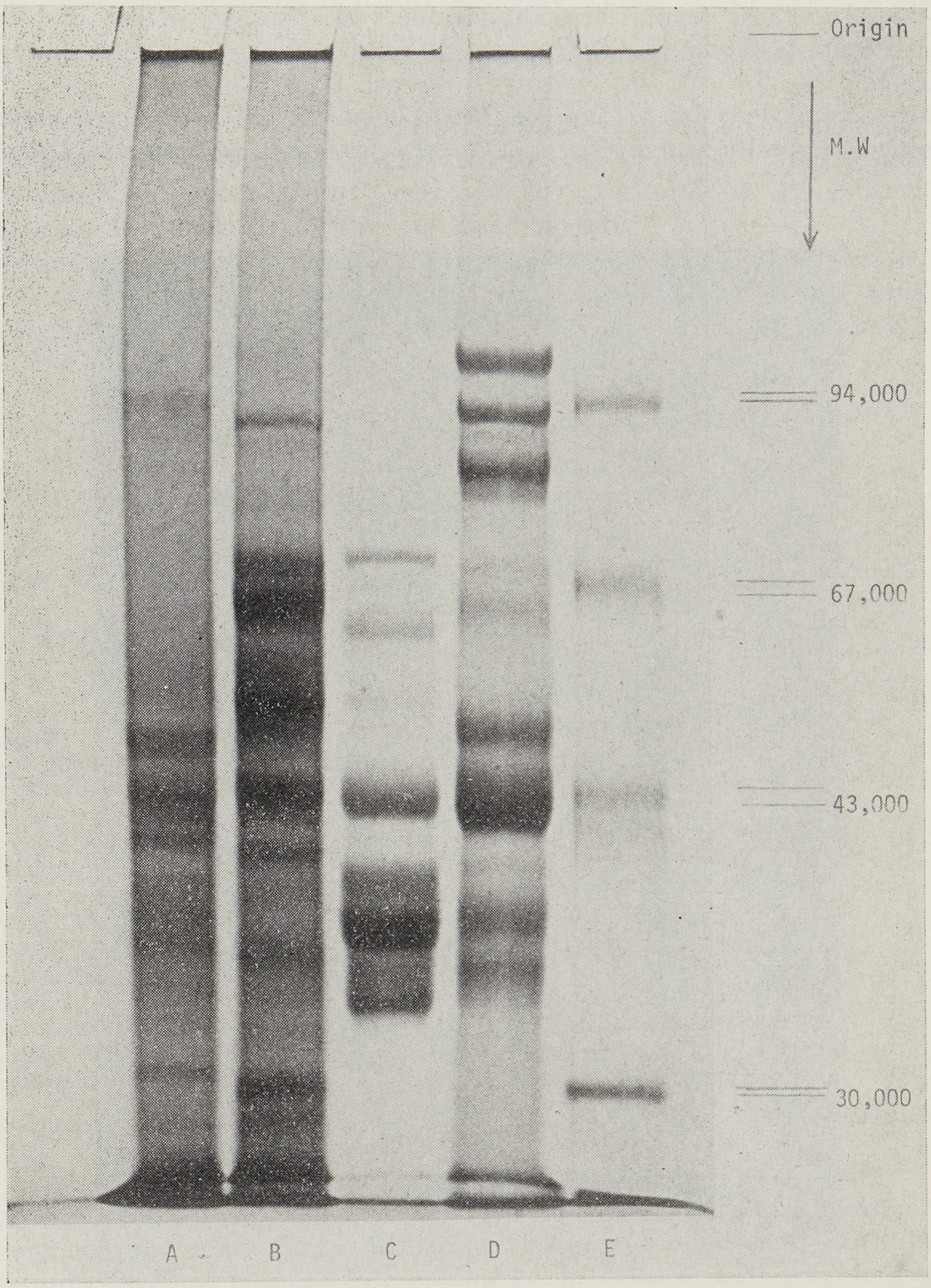

FIG. 2. — *Fractionation of protein samples by SDS-polyacryl-amide-gel electrophoresis after reduction with β-mercaptoethanol : A, glutelins from barley flour ; B, glutelins from wheat flour ; C, gliadins + glycoproteins V ; D, SDS-insoluble glutenins ; E, standard proteins : phosphorylase b (94,000), Albumin (67,000), Ovalbumin (43,000), Carbonic Anhydrase (30,000).*

no sugars. Upon resuspending the residue in SDS solution a gel was formed (Gel proteins). In a 0.1 N acetic acid solution containing $HgCl_2$ a gel was also formed. BIETZ *et al.* (1975) obtained the same fraction via a modified Osborne fractionation and named it acetic-acid-insoluble glutenin. In particular the electrophoretic composition has made us decide to classify the gel proteins as glutenins. Figure 2 shows the SDS-PAGE pattern of glutenin after reduction with 2-mer-

captoethanol. These results are comparable with those of KHAN and BUSHUK (1979). Figure 3 shows the eletrophoretic composition of reduced glutenins on a starch gel.

The alcohol extract of the gel layer consisted of proteinaceous material containing chemically bonded sugars. In the preceding publication (GRAVELAND

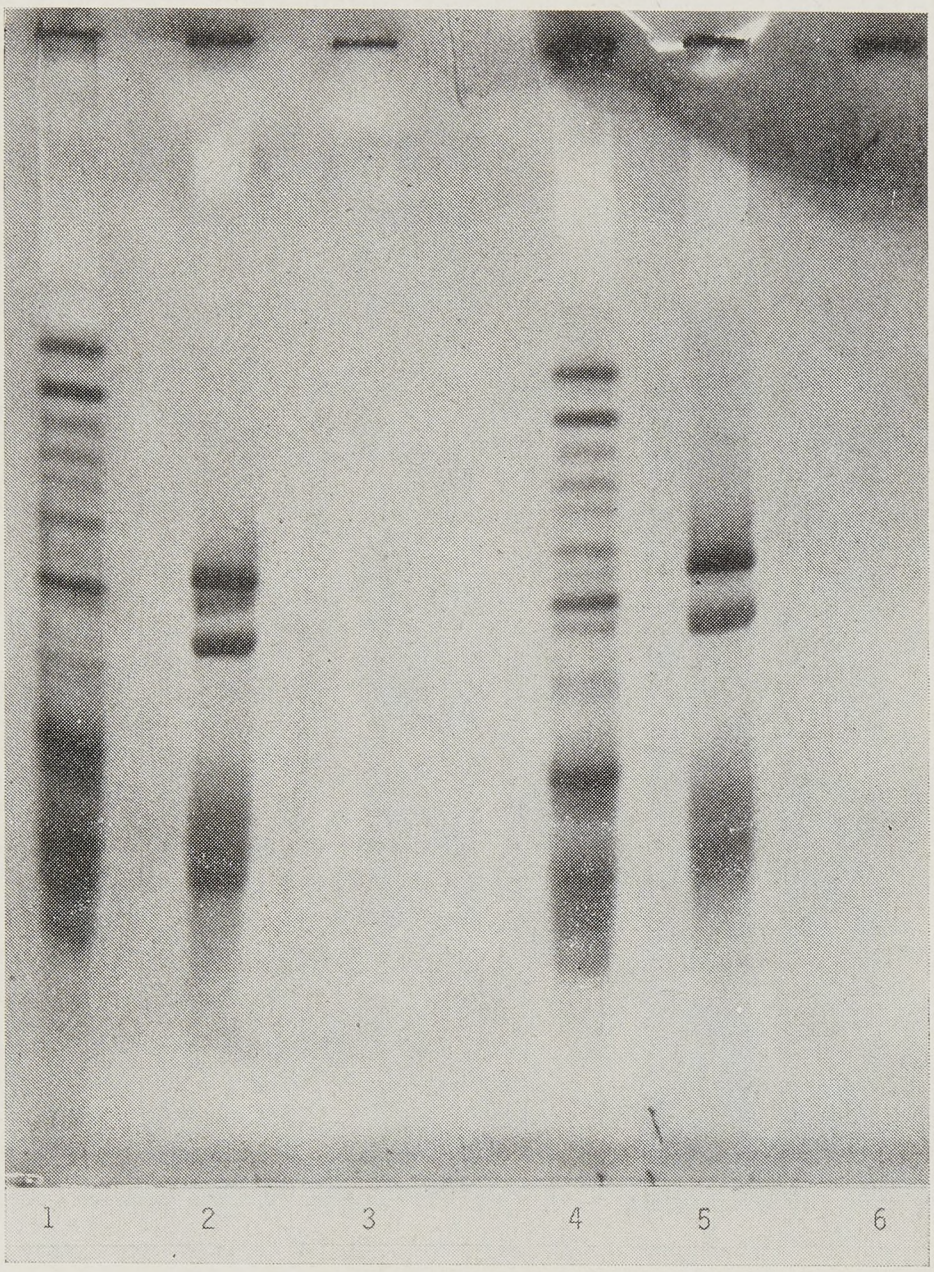

FIG. 3. — *Fractionation of protein samples by starch gel electrophoresis (9 p. 100 starch) after reduction with β-mercaptoethanol 1 and 4, gliadins + glycoproteins V (wheat); 2 and 5, SDS-insoluble glutenins (wheat); 3, glutelins (wheat); 6, glutelins (barley).*

et al., 1979) we have classified the whole gel layer as glycoproteins II. Now we have separated the gel layer into glutenins (gel proteins) and alcohol-soluble sugar-containing material. Therefore we have now classified this alcohol-soluble material as glycoproteins II. Table 1 gives the composition of the neutral sugars of these glycoproteins II. A stricking fact is the high percentage of galactose.

SDS-soluble, 70 p. 100 ethanol-insoluble material

SDS supernatant (Fig. 1) was fractionated by addition of ethanol. After centrifugation the precipitate was extracted with water. The water-soluble material contained water-soluble pentosans and glycoproteins IV, more research is needed for the characterisation of these fractions.

The precipitate remaining after the treatment with water was further extracted with a 0.03 M aluminium lactate buffer of pH 3.3 containing 1 M urea. The resulting supernatant contained proteins, which we have classified as globulins

TABLE 3

Distribution of nitrogen in the protein fractions
(percentages of total nitrogen)

Fraction	Wheat flour	Wheat flour dough	Barley flour	Gluten Ball
SDS-insoluble:				
Glycoproteins I	4	4	15	1
Glycoproteins II	2	2	3	2
Glutenins	28	14	7	70
SDS-soluble:				
Glutenins	6	18	10	4
Glutenins	13	13	38	0
Globulins / Glycoproteins IV	4	4	3	0
Gliadins / Glycoproteins IV	33	33	4	23
Albumins. / Glycoproteins VI	9	12	20	0

(GRAVELAND *et al.*, 1979). The precipitate was again extracted with a 0.1 N acetic acid solution containing 4 M urea and 0.1 p. 100 2-mercapto-ethanol. The supernatant contained reduced SDS-soluble gel proteins or SDS-soluble glutenins, identified by SDS-PAGE (see Fig. 4). The residue contained hydrophobic material insoluble in either 4 M urea, 0.1 N acetic acid or 70 p. 100 ethanol; this is why in our previous work (GRAVELAND *et al.*, 1979) we had classified this fraction as glutenins.

Because this fraction is comparable with the *glutelins* of barley we have now classified this fraction as glutelins (see Fig. 2 and Table 2). This fraction is insoluble in urea-lactate buffer of pH 3.3 and therefore cannot be separated on starch gel (see Fig. 3). The glutelins remained behind in the residue as acetic acid insoluble material using the modified Osborne fractionation (ORTH and BUSHUK, 1973). Moreover a gluten ball did not contain this fraction (Table 3). They were washing out as NaCl-insolbule material together with the starch and glyco-proteins I.

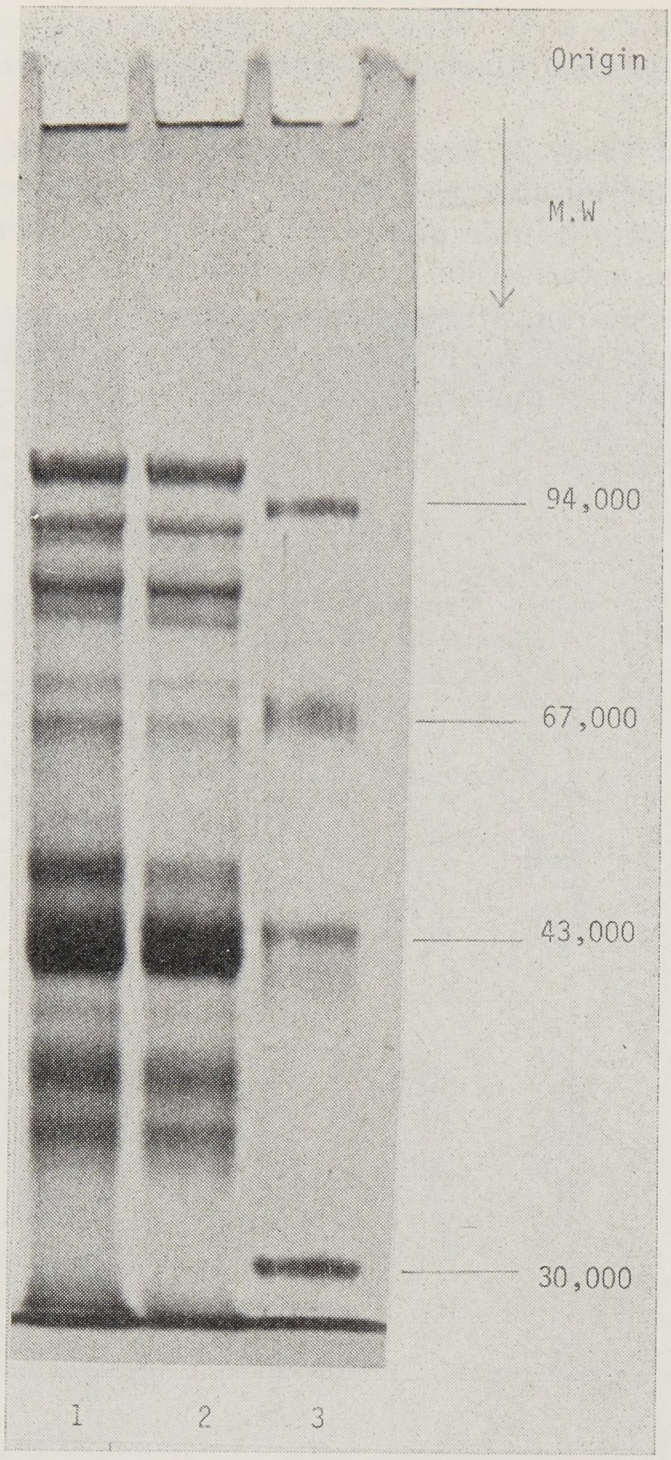

FIG. 4. — *Fractionation of protein samples by SDS-polyacrylamide-gel electrophoresis after reduction with β-mercaptoethanol: 1, SDS-soluble glutenins; 2, SDS-insoluble glutenins; 3, standard proteins.*

SDS-soluble and 70 p. 100 ethanol-soluble material

The ethanol SDS supernatant (300 ml) was reduced to about 80 ml and fractionated by addition of $(NH_4)_2SO_4$ to the final concentration of 10 p. 100. The precipitate contained gliadins and glycoproteins V (GRAVELAND *et al.*, 1979). The supernatant was cleaned of SDS by using a cation exchange resin AG2-X10 (column) and dialysed against distilled water.

Table 2 presents the amino acid composition of the fraction of gliadins + glycoproteins V, while Figures 2 and 3 show the electrophoretic composition of

this fraction. More research will be required to analyse this fraction in more detail. The quantities of the several protein fractions are presented in Table 3.

Conclusions concerning the fractionation of the wheat flour proteins

a) The SDS-insoluble light brown layer consisted of water-insoluble pentosans and glycoproteins I.

b) The SDS-insoluble gel layer consisted of gel proteins and glycoproteins II. The gel proteins are the same as the acetic acid insoluble proteins characterized by BUTAKI and DRONZEK (1979), KHAN and BUSHUK (1979), JEANJEAN and FEILLET (1978), DANNO *et al.* (1976). This fraction we have classified as SDS-insoluble glutenins.

c) The SDS-soluble, ethanol-insoluble fraction, which is soluble in 4 M urea after reduction with 2 mercapto-ethanol, has the same amino acid composition and the same electrophoretic pattern as the SDS-Insoluble glutenin. We have classified it as SDS-soluble glutenins.

d) The SDS-soluble, 70 p. 100 ethanol-insoluble fraction, insoluble in 4 M urea, is similar to the glutelins from barley. We therefore have classified this fraction as glutelins.

Proteins of wheat flour dough

During dough-mixing the SDS-insoluble gel proteins or glutenins are converted into smaller fragments which are soluble in SDS. The mixing results, moreover, in the glycoproteins II fraction being released from the gel layer complex. In consequence of these processes the gel layer diminishes during mixing.

Figure 5 shows that the conversion of gel proteins to SDS-soluble material depends on the concentration of oxygen. It has been established that the conversion of gel proteins is due to SS bonds being split. The number of SS bonds in this fraction decreases, while the number of SH groups increases. The gel

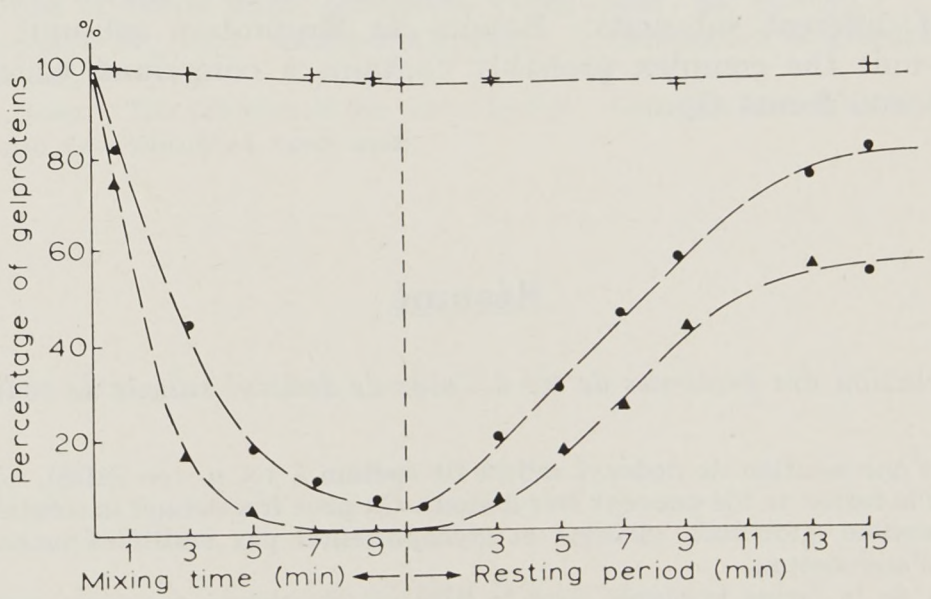

FIG. 5. — *Breakdown of the gel proteins during dough-mixing, and re-formation during the resting period.* + *dough mixed under nitrogen,* ● *under air,* ▲ *under oxygen.*

proteins contain approximately 50 μmole SS per gram of material. During mixing under air and oxygen, 20 and 24 p. 100 of the SS bonds were cleaved respectively. When mixing under nitrogen only 4 p. 100 of the SS bonds were reduced to SH.

A study (GRAVELAND *et al.*, 1980) conducted to identify the component responsible for the reduction of SS-bonds has established that superoxide anions (O_2^-.) were involved in this reaction.

During dough-mixing under air or oxygen the superoxide anion is formed. This anion reduces an Mn^{3+}-protein compound to a Mn^{2+}, which in turn reduces a $2Cu^{2+}$-protein compound to $2Cu^{+}$. This $2Cu^{+}$-protein compound is capable of reducing an SS bond of glutenin to SH. The transfer of the electron from O_2^-. to an SS bond has been represented in Figure 6.

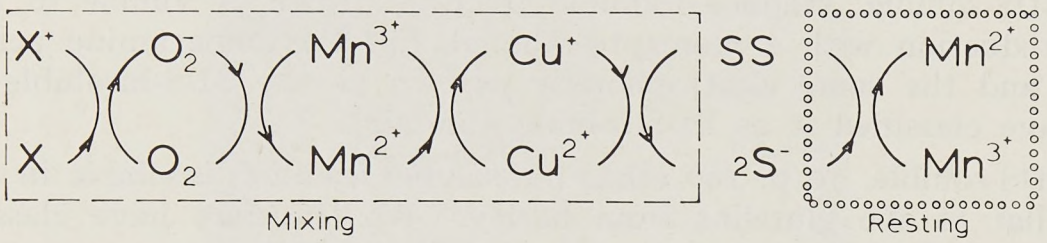

FIG. 6. — *Reduction of SS bonds during dough-mixing and the oxidation of SH groups during the resting period.*

Another interesting phenomenon in the fact that during the fermentation period after mixing the dough, the gel layer re-forms from the reaction products formed from gel proteins during the mixing. This re-formation of the gel layer under air or oxygen during the fermentation period proceeded slower than the breakdown. After 10 mn mixing under air, 80 p. 100 of the original gel layer reformed and after 10 mn mixing under oxygen 60 p. 100.

From a fundamental study it appeared that the re-formation of the gel layer was based on the oxidation of SH groups. The oxidation of SH groups is brought about by Mn^{3+} ions. From the extraction experiments with isopropanol at 70 °C and the EPR measurements we suspect that we have here a protein complex composed of different sub-units. Besides an Mn-protein sub-unit and a 2Cu-protein sub-unit the complex probably contains a compound which is soluble in methanol and forms O_2^-..

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Résumé

Extraction des protéines de blé à l'aide de dodecyl sulfate de sodium

A l'aide d'une solution de dodecyl sulfate de sodium à 1.5 p. 100 (SDS), 65-70 p. 100 de l'azote total de la farine de blé peuvent être dissous. On peut fractionner la solution en glutéline pentosane, albumine, globuline, gliadine et glycoprotéines par additions successives d'alcool et de sulfate d'ammonium.

Le résidu de la farine insoluble dans le SDS est fractionné par ultra-centrifugation en une couche inférieure d'amidon, une couche supérieure de matériel gélatineux et une couche intermédiaire brune claire (*J. Sci. Fd. Agric.*, **30** (1979), 71-84). Si l'on veut remettre en sus-

pension dans le SDS cette couche intermédiaire, et si l'on centrifuge, il en résulte une couche blanche et une couche brune, contenant toutes deux de hauts pourcentages de pentoses; la couche brune contient en outre 10 p. 100 de matériel protéique que l'on appelle glycoprotéines I. La couche supérieure, ou couche de gel, peut être séparée de façon similaire en « protéines de gel SDS » ou gluténines et une fraction soluble appelée glycoprotéines II.

Dans cette communication, nous discutons les propriétés physiques et chimiques des fractions insolubles dans le SDS et des glutélines solubles dans le SDS. Les « protéines de gel SDS » ou gluténines jouent un rôle important dans la panification : pendant le pétrissage, elles sont dissociées en substances solubles dans le SDS par suite de la rupture de leurs ponts disulfures et après le pétrissage il y a reformation de « protéines de gel SDS ». La rupture des ponts disulfures est causée par des électrons issus des anions superoxyde (O_2^{-}.) et transportés par un complexe Mn /Cu-protéines. Un complexe Mn^{3+}-protéines est impliqué dans la reformation des ponts disulfures par oxydation à partir des groupes SH (*Biochem. Biophys. Res.* Comm. sous presse).

Mots clés : *blé - gel protéique - gluténine - protéine* - SDS.

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Extraction of wheat proteins with salts of fatty acids and their electrophoretic characterization

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Summary

Wheat flour proteins are quantitatively solubilized in distilled water containing Na-salts of fatty acids. The efficiency of the solubilization depends upon the amount of soap used, the pH of the solution has not an important role. The solubilization of protein groups (albumins, globulins, gliadins, glutenins), as shown by electrophoresis, does not occure simultaneously. There are important differencies also in the solubility of protein fractions within each group especially in the case of gliadins and glutenins. More specific interactions or links between some protein fractions could also be established. High Molecular Weight (M.W.) subunits (> 90,000) are solubilized only with higher amount of soaps. Results are not similar when flours from different wheat varieties with different bread-making quality are used i.e. the solubility of high M.W. glutenin subunits and of some gliadins seems to be related to the bread-making quality of flour.

Key words : *wheat, proteins, extraction, soaps, electrophoresis, hydrophotric interactions.*

Introduction

It has been shown that glutenin can be solubilized in distilled water in the presence of sodium salts of fatty acids (KOBREHEL and BUSHUK, 1977; KOBREHEL and BUSHUK, 1978; WASIK *and al.*, 1979; HAMAUZU *and al.*, 1979). This technique does not disrupt disulfid bonds (WASIK *and al.*, 1979; HAMAUZU *and al.*, 1979). The solubilization of glutenin can be explained by the hydrophobic interactions between soap molecules and the polypeptides chains (KOBREHEL and BUSHUK, 1977; WASIK *and al.*, 1979). These earlier results support the hypothesis of KASARDA *and al.* (1976) that the disulfide bonds within glutenin are intramolecular rather than intermolecular.

In the present work we applied this solubilization technique to the flour. Results reported here give additional informations on the structure of wheat proteins as well as the function of these proteins.

Materials and methods

Several French wheat varieties (*Triticum aestivum*) with different bread-making properties were analyzed. Flours from these varieties were obtained using a laboratory mill (CHOPIN).

Proteins were solubilized by mixing 0-200 mg of soap (Na-dodecanoate, Na-hexadecanoate, Na-octodecanoate) or 3-300 µl of hexanoic acid, 2 g of flour, and 20 ml of distilled water in centrifuge tubes, stirring overnight at room temperature and centrifuging for 15 mn (32,000 × g) at 4 °C. The supernatants were used to determine the amounts of solubilized proteins (Kjeldahl method) or for electrophoresis.

Second and third extractions were performed with distilled water without additional amount of soap or fatty acid.

Polyacrylamide gel electrophoresis (PAGE) were performed in sodium dodecyl sulfate-Tris-borate buffer at pH = 8.9 (BIETZ and WALL, 1972), and in aluminium-lactate buffer at pH = 3.2 according to KOBREHEL and FEILLET (1971) and in the conditions described by BUSHUK and ZILLMAN (1978).

Results and discussion

Solubilization of Proteins

Figure 1 shows the curves of wheat flour protein solubility obtained with three soaps, Na-dodecanoic, Na-hexadecanoic, Na-octodecanoic and with hexanoic acid. Distilled water dissolves about 20 p. 100 of total flour protein. Addition of soap or fatty acid increases the amount of solubilized proteins. However, at very low soap concentrations the amount of solubilized protein decreases slightly. At the optimal soap concentrations, the total amount of solubilized protein of three successive extractions varies between 97 and 100 p. 100. Most of the proteins are solubilized during the first extractions. Using very high amounts of soap or fatty acid the quantity of solubilized protein decreases. The three soaps give similar results. Hexanoic acid is much less efficient as a solubilizing agent than the soaps.

The quantitative solubilization of wheat flour proteins with soaps is not surprising if we consider that glutenins can be solubilized in similar conditions. The mechanism of this solubilization is not yet known and it is probably the same in both cases. These results suggest that about 80 p. 100 of wheat flour proteins which are solubilized with higher amount of soaps, are aggregated through non-covalent bonds. A certain amount of soap is needed to disrupt these aggregates, when this amount is reached a sharp, nonlinear increases in protein solubility can be noticed. Hydrophobic interactions should play an important role in these aggregates since soaps exert their action mainly on these bonds (WASIK *and al.*, 1979) without affecting covalent bonds (HAMAUZU *and al.*, 1979). At very high soap concentrations, interactions between soap molecules become more important and consequently the interactions between protein residues and soap molecules decrease gradually. A similar phenomenon occures when simultaneously

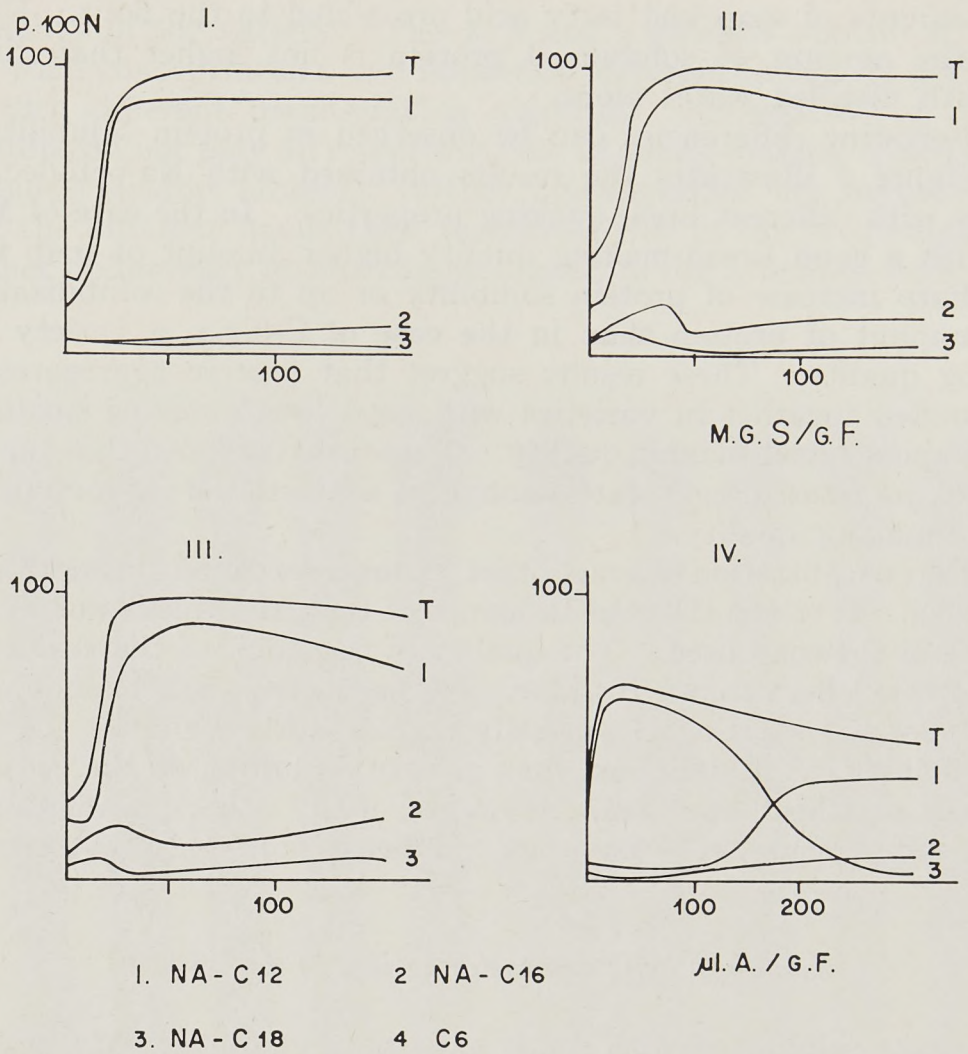

FIG. 1. — *The solubility of wheat flour proteins* (var. HARDI) *in distilled water with increasing amounts of Na-dodecanoate (I), Na-hexadecanoate (II), Na-octodecanoate (III), and of hexanoic acid (IV) (mg of soap or µl of acid per g of flour). 1, 2, 3: first, second and third extraction. T = the total amount of solubilized protein in percentage.*

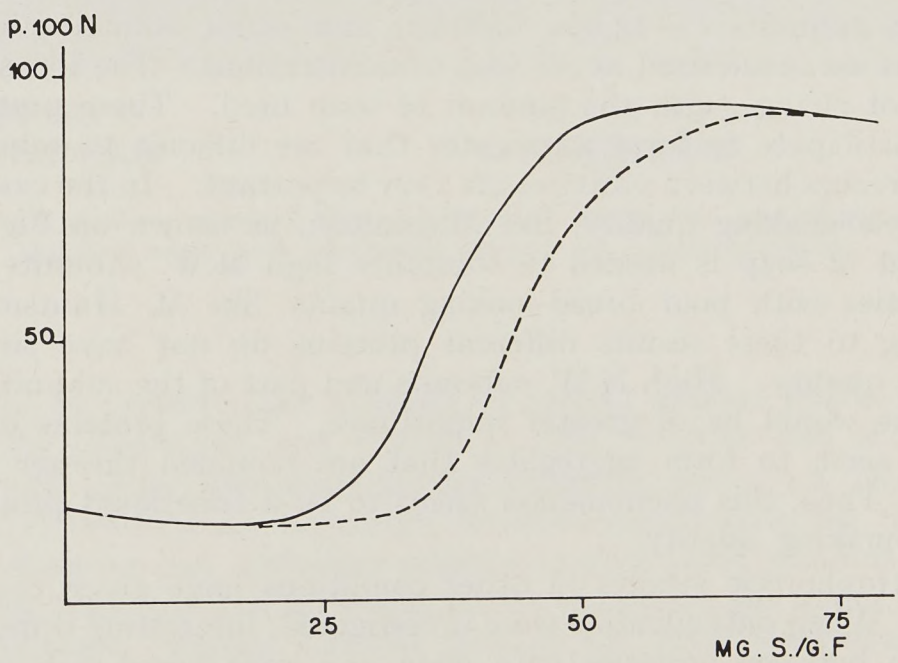

FIG. 2. — *Comparison of the solubility of wheat flour proteins of two varieties with different bread-making quality (Magdalena and C-6.2.4) in distilled water with increasing amounts of Na-octodecanoate.*

increasing amounts of soap and fatty acid are added to the flour. Under these conditions, the amount of solubilized protein is not higher than the amount extracted with distilled water alone.

Very interesting differencies can be observed in protein solubility between varieties. Figure 2 illustrates the results obtained with Na-octodecanoate for two varieties with different bread-making properties. In the case of Magdalena, a variety with a good bread-making quality higher amount of soap is required up to the sharp increase of protein solubility or up to the solubilization of the maximum amount of protein than in the case of C-6.2.4, a variety with poor bread-making quality. These results suggest that protein aggregates are more strongly bounded together in varieties with good bread-making quality than in varieties with poor bread-making quality. Thus it is concluded that the capability of wheat flour proteins to aggregate strongly is a functional characteristic related to the bread-making quality.

Beside the concentration of soap, other parameters can influence the solubility of proteins also. It seems that the influence of the pH value is not as important as the nature of the soap used. The quality of the flour, of the variety, has also a more important effect than pH value. Actually with higher amounts of soap, the pH value of the solution is generally higher, but for similar pH values the amounts of solubilized protein can vary greatly according to the soap used and to the variety of wheat analyzed. However, many other parameters (ratio of water/flour, temperature, etc.) have not yet been thoroughly investigated.

Electrophoretic characterization of proteins

The proteins solubilized with soaps have been characterized under different electrophoretic conditions. Figure 3 shows the electrophoretic patterns of proteins using SDS-Tris-borate buffer at pH = 8.9. In these conditions proteins are separated according to their molecular weight (M.W.). As shown on this figure, the high M.W. subunits, as well as some of the polypeptides having about 45,000 daltons, are solubilized at higher soap concentrations. Most of the low M.W. protein subunits (~ 13,000 daltons) and other subunits having about 60,000 daltons are solubilized at all soap concentrations. The intensity of these bands does not change with the amount of soap used. These protein subunits would not participate to form aggregates that are difficult to solubilize.

The differences between varieties are very important. In the case of varieties with good bread-making quality, like Magdalena, as shown on Figure 3, much higher amount of soap is needed to solubilize high M.W. subunits than in the case of varieties with poor bread-making quality like M. Huntsman.

According to these results different proteins do not have similar role in bread-making quality. High M.W. subunits and part of the subunits with about 45,000 daltons would be of greater importance. These proteins depending on the varieties seem to form aggregates that are bounded through noncovalent interactions. Thus, this phenomenon seems to be a functional property related to the bread-making quality.

The electrophoretic studies in other conditions have given complementary information. When only gliadins were investigated, interesting differencies could be seen again between varieties with good and poor bread-making properties. An example is presented on Figure 4. As shown on this figure, the solubilization of ω-gliadins does not relate to neither the amount of soap used, nor the bread-

making quality of the variety. The α, β and γ gliadins appear at both higher and lower soap concentrations and important differencies can be noticed between varieties with different bread-making qualities. Consequently it is concluded that ω-gliadins do not play an important role in bread-making quality. Concerning α, β and γ gliadins however, further investigations are necessary to assign to each of the different bands their functional role in the bread-making quality. These, rather preliminary results can be related to the attempts made by different

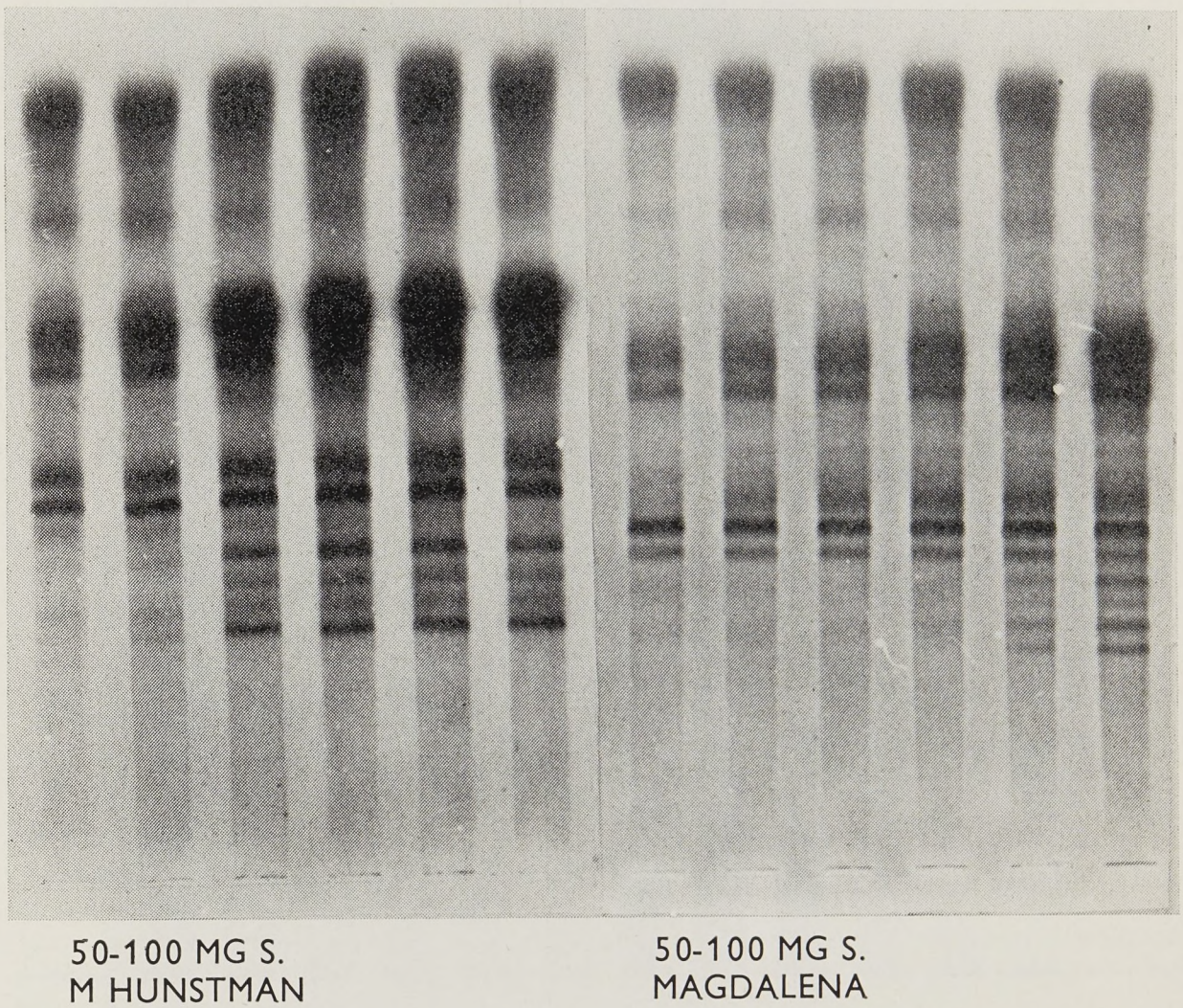

FIG. 3. — *PAGE patterns of wheat flour proteins var. Magdalena and Maris Huntsman, solubilized in distilled water with increasing amount of Na-octodecanoate. Tris-SDS buffer, pH = 8.9.*

authors trying to correlate the presence of specific gliadin bands to the bread-making quality (BRANLARD and ROUSSET, 1980; SOZINOV *and al.*, 1974).

With PAGE using Aluminium-lactate buffer at pH = 3.2 (KOBREHEL and FEILLET, 1971) proteins are separated simultaneously according to their charge and M.W. Albumins and globulins migrate readily while gliadins remain close to the starting base line and glutenins do not enter the gel under these electrophoretic conditions. Figure 5 shows that at low soap concentrations most of the albumins and globulins are solubilized. Gliadins generally require higher amounts of soap in order to be solubilized. However some of the gliadins, probably ω-gliadins as indicated above, are solubilized using relatively low soap

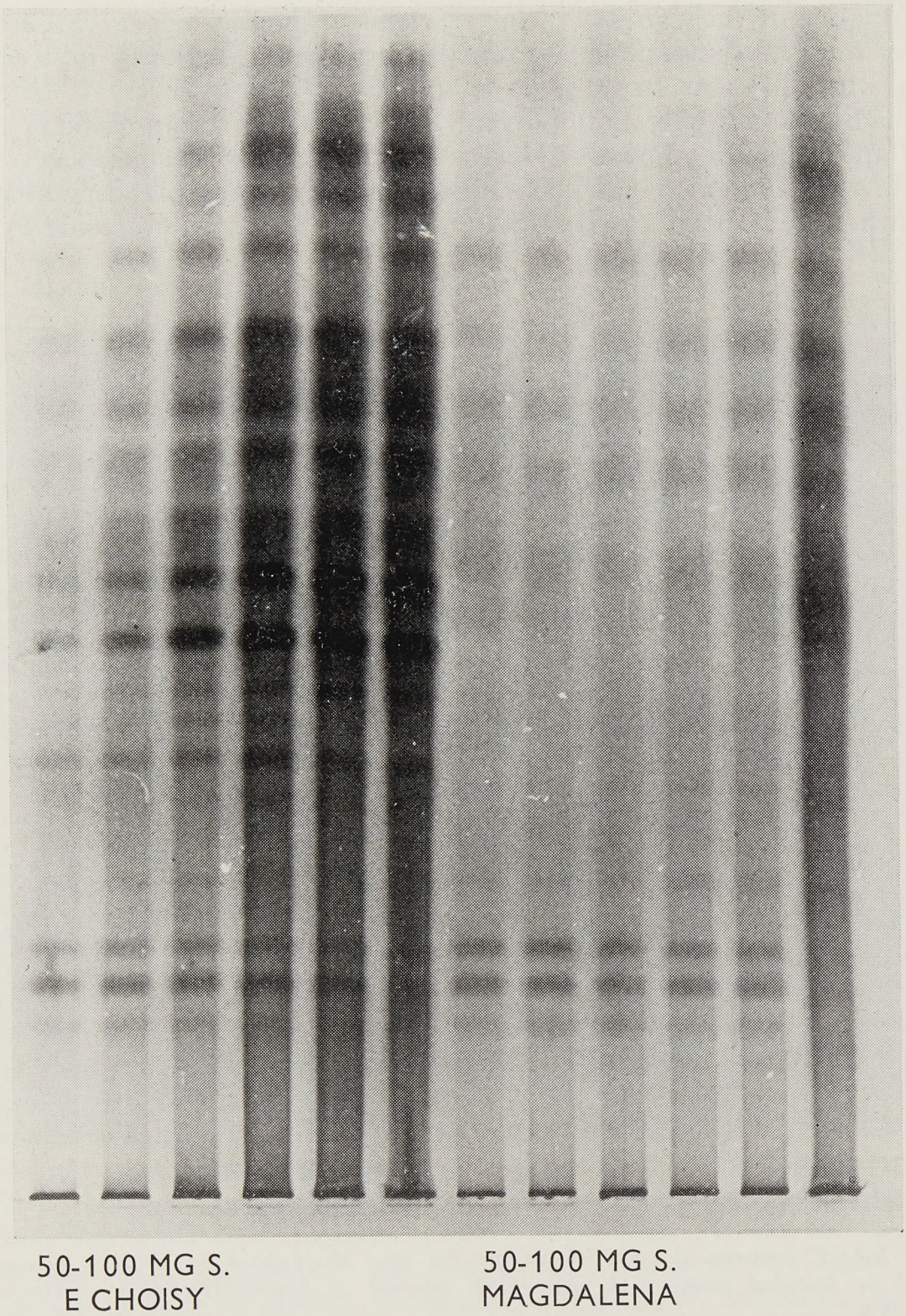

FIG. 4. — *PAGE patterns of gliadins var. Magdalena and Étoile de Choisy, solubilized in distilled water with increasing amounts of Na-octododecanoate, Aluminium-Lactate buffer, pH = 3.2.*

concentrations especially in the case of varieties with poor bread-making quality. On Figure 5, Clement is a variety with poor and Magdalena with a good bread-making quality. In the case of the variety Magdalena instead of well distinguishable protein bands, patterns are shown to be very streaky. Under these electrophoretic conditions, this streakiness happens generally in the case of varieties with better breadmaking qaulity. It may be due to the stronger aggregation of proteins in these varieties.

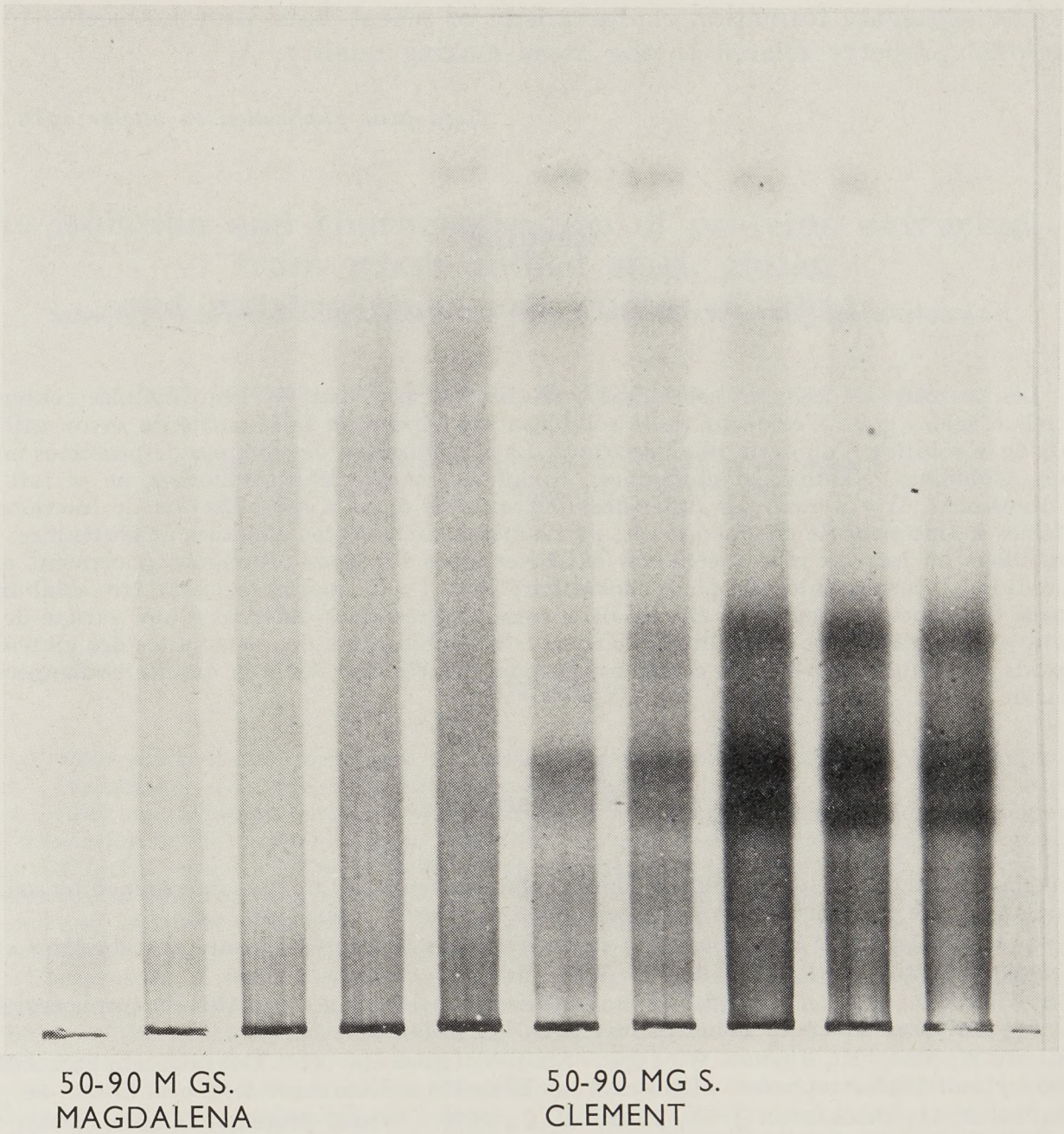

FIG. 5. — *PAGE patterns of wheat flour proteins var. Magdalena and Clement, solubilized in distilled water with increasing amounts of Na-octodecanoate, Aluminium-Lactate buffer, pH = 3.2.*

Conclusions

Wheat flour proteins can be quantitatively solubilized with distilled water containing Na-salts of fatty acids.

Wheat flour proteins seem to be composed of proteins that are easy to solubilize with distilled water and of aggregated proteins solubilized with higher or lower soap concentrations depending on the quality of the variety.

The easily solubilized proteins would be the albumins, globulins and some of the gliadins, namely ω-gliadins.

Protein aggregates would be composed mainly of glutenins and α, β and γ-gliadins.

The aggregate formation characteristic of wheat flour proteins seems to be functional property related to the bread-making quality.

Reçu pour publication en octobre 1980.

Résumé

Extraction des protéines du blé et leur caractérisation par électrophorèse

Les protéines de blé sont solubilisées quantitativement dans de l'eau distillée contenant des sels d'acides gras. L'efficacité de la solubilisation dépend de la quantité de savon utilisée, le pH de la solution n'a pas un rôle important. La solubilisation des groupes de protéines (albumines, globulines, gliadines et gluténines), comme le montre l'électrophorèse, ne se fait pas simultanément. Il y a aussi des différences importantes dans la solubilisation de fractions de protéines à l'intérieur de chaque groupe, particulièrement pour les gliadines et gluténines. Des interactions ou liaisons plus spécifiques entre certaines fractions protéiques pourraient aussi se produire. Des sous-unités de poids moléculaire élevé (> 90,000) ne peuvent être solubilisées qu'avec des concentrations plus élevées de savons. Les résultats diffèrent d'une variété de blé à l'autre en relation avec la qualité boulangère : la solubilisation des sous-unités des gluténines de poids moléculaire élevé et de certaines gliadines semble être liée à la qualité boulangère de la farine.

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The isolation and characterisation of proteins extracted from whole milled seed, gluten and developing protein bodies of wheat

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Summary

Various solvents have been used to isolate the protein components of whole milled wheat flour. Preliminary defatting with water saturated butanol or 2 : 2 : 1 (v/v) ethanol : diethyl ether : water decreased the amount of N extracted in the salt soluble fraction and increased that remaining in the residue fraction. The amount of N extracted by alcoholic solvents after prior extraction with 0.5 M NaCl increased as a function of the alcohol used (50 p. 100 (v/v) propan-1-ol > 50 p. 100 propan-2-ol = 70 p. 100 ethanol) the presence of 1 p. 100 2-mercaptoethanol and increased temperature. The maximum amount of N (ca 60 p. 100 of total seed-N) was extracted by 50 p. 100 propan-1-ol, 1 p. 100 2-mercaptoethanol, 1 p. 100 acetic acid at 60 °C.

The proteins of washed gluten were examined by solubilizing them in 0.01 M acetic acid, 6 M urea, 0.055 M cetyltrimethyl ammonium bromide, a treatment which left less than 5 p. 100 of the N in the pellet after centrifuging the extract at 20,000 × g for 30 mn. The proteins were further fractionated into size classes by chromatography on controlled pore glass columns (pore diam. 700 Å) and two fractions obtained — one of high molecular weight and a broader peak eluting within the volume of the column. The high molecular peak was not obtained when the proteins were reduced with mercaptoethanol. A similar size distribution of proteins was obtained when the same techniques were used to characterise the proteins present in the protein bodies of developing wheat endosperms.

The various protein fractions obtained were compared by one- and two-dimensional electrophoretic techniques and show that the polypeptide components of the propan-1-ol : mercaptoethanol : acetic acid soluble fraction of milled grain, of the washed gluten, and of protein bodies from developing endosperms, are essentially similar. We consider that these polypeptides comprise the prolamin storage protein fraction of wheat seed.

Key words : *wheat, proteins, composition, protein bodies.*

Introduction

The objects of current work in our laboratory are to:

(1) Provide as completely as possible a balance sheet for the distribution of N and S in the different protein fractions of wheat seed.

(2) Understand the physiological roles and sub-cellular organisation of the protein groups that we are studying — particularly to define which are the storage proteins of the starchy endosperm (i.e. not aleurone) of wheat.

(3) To correlate the first two aims with the technological properties of the proteins particularly in regard to baking quality.

We have therefore used various techniques to isolate, extract and characterise the component polypeptides present in wheat endosperm. In this work we have used Osborne's definition of a prolamin as the fraction soluble in ethanol (70-80 p. 100), or in other alcohols (OSBORNE, 1924), with one additional qualification. This is that if a polypeptide is completely soluble in an alcohol only in the presence of a reducing agent it may still be classified as a prolamin. This seems to us a reasonable addition and one which would not have occurred to Osborne, since the discovery of disulphide bonds in proteins is generally credited to HOPKINS (1925). However, we believe this definition is of limited use because it is relatively imprecise, it does not involve criteria based on the electrophoretic properties of the protein, on their physiological function, on the sub-cellular location, or on their genetics, all of which are of importance in the characterisation of a protein or protein class (see MIFLIN and SHEWRY, 1979, for a further discussion of these points). We have used the term glutelin as the generic term for the fraction remaining after all of the albumin, globulin and prolamin have been removed for which the specific term in wheat is glutenin (OSBORNE, 1924; BAILEY, 1944). Our definition of the prolamin and glutelin fractions of wheat (and other cereals) is not comparable to the modified and limited versions of the terms, based on incomplete extraction in 70 p. 100 ethanol or on molecular weight separations, that have been widely used in the literature and which we have argued elsewhere (MIFLIN and SHEWRY, 1979; SHEWRY *et al.*, 1978*b*) are inappropriate.

Methods

The extraction of protein fractions from whole milled wheat grain

(a) *Defatting*

Wheat grain (cv. Highbury) was milled in a Glen Creston Hammer Mill to pass an 0.5 mm sieve. We then tested the effect of defatting the meal prior to extraction of the protein. The solvents used were water-saturated butan-1-ol or ethanol: diethyl ether: water (2 : 1 : 1 by volume) and 2.0 g of flour were extracted twice with 20 ml solvent per extraction.

(b) *Salt-soluble fractions*

Albumins, globulins and non-protein nitrogen were removed together to give a " salt-soluble fraction ". The rationale is that the division between albumins and globulins is a fine one and probably not particularly useful, except in specific cases. On a physiological basis these proteins may also be expected to be chiefly metabolic proteins. Although storage globulins may exist in the embryonic axis, the scutellum and the aleurone layer, they are unlikely to form a major proportion

of the total-N. Thus defatted or untreated meal was extracted sequentially with 0.5 M NaCl (2 × 20 ml) and water (1 × 15 ml) at 4 °C and the extracts bulked.

(c) *Prolamin fraction*

The residue from the salt-soluble extract was then extracted using one of the four following solvents: 70 p. 100 (v/v) ethanol, 50 p. 100 (v/v) propan-1-ol (with or without 1 p. 100 acetic acid) and 55 p. 100 (v/v) propan-2-ol. All four were also used with the addition of 1 p. 100 (v/v) 2-mercaptoethanol as a reducing agent. For 2 g original weight of flour one extraction with 20 ml and two extractions with 15 ml were made at either 4 or 60 °C. Extraction was carried out for 30 mn using rapid mechanical stirring in a device based on that of Kφie and Nielsson (1977). The extracts were centrifuged at 25,000 × g for 45 mn and the supernatants bulked. The pellet from the final extraction was termed the residue. Proteins were recovered from the supernatants by dialysis against distilled H_2O overnight, concentration *in vacuo* and freeze-drying.

Isolation of protein bodies

The methods of Miflin *et al.* (1980) were used. Developing endosperms of wheat (cv. Highbury) were chopped in 50 mM tricine pH 7.5, 100 mM KCl, 5 mM EDTA and 0.3 M sucrose and then filtered through four layers of cheesecloth. The filtrate was then layered on a 16-60 p. 100 (w/w) linear sucrose gradient made up in 50 mM tricine pH 7.5, 100 mM KCl and 1 mM EDTA. The gradients were centrifuged at 25,000 rpm in a Beckman SW 27 rotor for 2.5 h and then fractionated using an ISCO density gradient fractionator Model 625. Fractions from a density of 1.24 to 1.28 g cm^{-3} were collected and protein bodies recovered by diluting the sucrose content to 20 p. 100 w/w and centrifuging for 30 mn at 50,000 × g.

Extraction of proteins from gluten and protein bodies

Gluten was prepared from whole wheat (cv. Highbury) meal and washed with 0.5 M NaCl followed by water prior to lyophilisation. Dried gluten was finely ground and 500 mg aliquots extracted by stirring with 15 ml 6 M urea, 0.055 M cetyltrimethyl ammonium bromide (CTAB), 0.01 M acetic acid at 40 °C. After 18 h, solutions were clarified by centrifugation for 2 h at 28,000 × g at 30 °C.

Protein body preparations were washed with water and solubilised as described above.

Controlled-pore glass chromatography

Five ml aliquots of the clarified gluten extract were applied directly to a column (1.1 × 90 cm) of controlled pore glass (700 Å pore diameter, 120-200 mesh) (Sigma Chemical Co. Ltd.) equilibrated and eluted at 35 °C with 6 M urea, 0.055 M CTAB, 0.01 M acetic acid at a flow rate of 20 ml/h. The column effluent was monitored at 278 nm and 3.5 ml fractions collected and pooled as appropriate.

Protein body extracts (0.5 ml aliquots) were fractionated on a smaller column (0.7 × 40 cm) of controlled-pore glass eluted at 35 °C with extraction solvent at a flow rate of 10 ml/h. In this case the fraction size was 0.5 ml.

Pooled fractions were extensively dialysed against distilled water, at room temperature for the initial 24 h and at 4 °C after that, and lyophilised. Prior to pyridylethylation freeze-dried material was washed with anhydrous methanol in order to remove bound detergent.

Gel electrophoresis

Prior to gel electrophoresis all proteins were alkylated using 4-vinylpyridine (SHEWRY *et al.*, 1978 *b*; FRIEDMAN *et al.*, 1970). Isoelectric focusing (IEF), sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and two dimensional IEF/SDS-PAGE were carried out essentially as described pre-previously (SHEWRY *et al.*, 1978 *a*) except that 12.5 p. 100 acrylamide gels were used for SDS-PAGE.

Results

Extraction of proteins from milled whole grain

Although the detailed results of this study will be presented elsewhere they may be summarised as follows. The effects of defatting are given in Table 1 which shows that both solvents extract a small proportion of the total N and affect the relative amounts of N extracted in the salt soluble fraction. The decreased solubility of the albumins and globulins after defatting is probably due to denaturation of the protein by the solvent; as a consequence of this the proteins thus appear in the residue or glutelin fraction. In contrast, defatting has virtually no effect on the amount of N extracted by the gliadin solvent.

TABLE 1

Effect of defatting on subsequent extraction of N from whole milled wheat grain (cv. Flinor)

Extracting solvent	Temp of extraction (° C)	Defatting solvent		
		None	Ethanol/Ether/Water (% distribution of recovered N)	Water-saturated butan-1-ol
Defatting solvent . . .	4	—	2.7	1.9
0.5 M NaCl. } Water }	4	33.6	24.8	21.9
50 % propan-1-ol + acetic acid (1 %) .	60	52.1	51.0	51.7
Residual N.		14.3	21.5	24.5

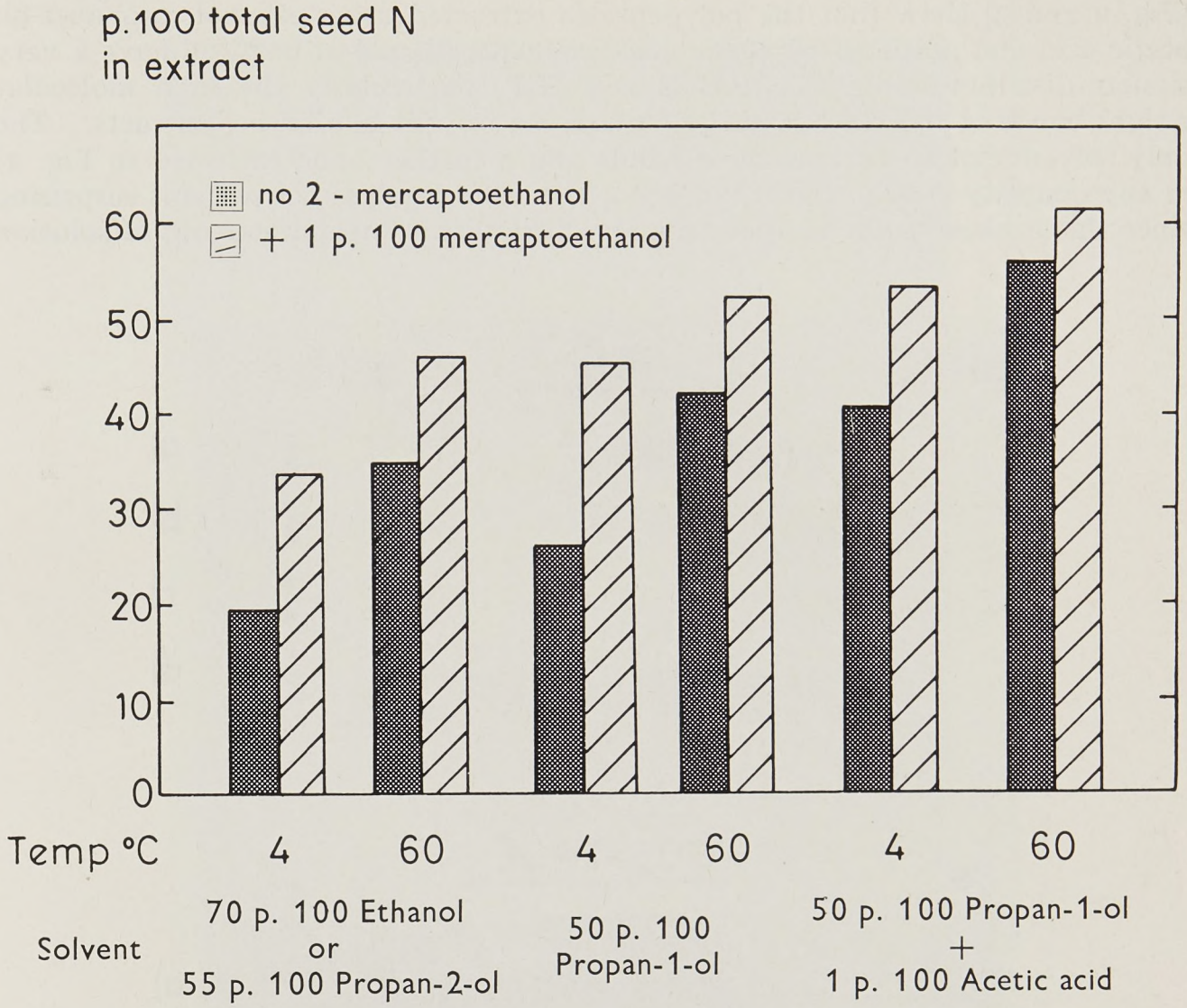

FIG. 1. — *The extraction of N from whole milled wheat grain (cv. Flinor) with different solvent systems at 4 and 60 °C.*

These conclusions differ slightly from previous results by WREN and NUTT (1967) and by CHARBONNIER (1973) who found lipid extraction lowered the solubility of gliadins.

Different alcoholic solvents were tested in the presence or absence of mercaptoethanol and at different temperatures and the amount of N extracted measured. The results obtained in one comparison with one variety are shown in Figure 1 — they are generally representative of others obtained with different varieties and in replicate extractions. As expected, less N was extracted, at lower temperatures and ethanol proved to be less effective than propan-1-ol (cf. DILL and ALSBERG, 1925, and other work quoted in BAILEY, 1944); propan-2-ol was little different from ethanol. As previously shown for barley (BISHOP, 1939; LONTIE *et al.*, 1953; LONTIE and VOETS, 1956; SHEWRY *et al.*, 1978) and for maize (FOSTER *et al.*, 1950; MOUREAUX and LANDRY, 1968; SODEK and WILSON, 1971) the addition of a reducing agent (in this instance 1 p. 100 2-mercaptoethanol) increases the amount of N extracted; the proportional effect is less the more efficient the extraction conditions are in its absence.

To investigate the polypeptide nature of the extracted proteins these were alkylated and subjected to one- and two-dimensional electrophoresis. The results

(Fig. 2 and 3) show that the polypeptides extracted using ethanol, propan-1-ol: acetic acid and propan-1-ol: acetic acid: mercaptoethanol at 60 °C all have a very similar distribution on SDS-PAGE and IEF, particularly the high molecular weight bands of 100 kilodaltons (kd) which are present in all three extracts. The only solvent not to extract these bands and a further band (arrowed in Fig. 2) in any quantity is 70 p. 100 ethanol at 4 °C. This result is not perhaps surprising since this is close to the temperature at which gliadin precipitates out of solution

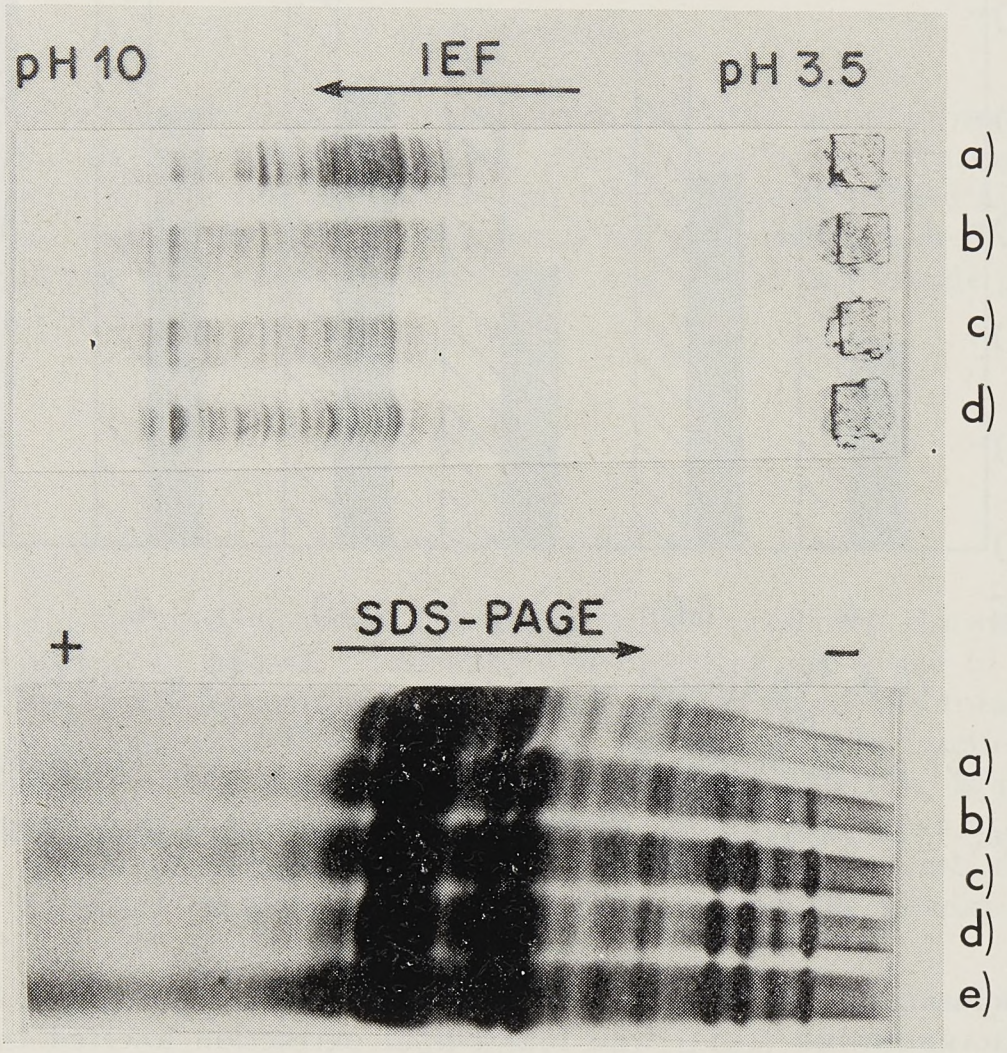

FIG. 2. — *One dimensional IEF and SDS-PAGE of proteins extracted from whole milled grain (cv. Highbury) by (a) 70 p. 100 ethanol at 4 °C, (b) 70 p. 100 ethanol at 60 °C, (c) 50 p. 100 propan-1-ol : 1 p. 100 acetic acid, (d) 50 p. 100 propan-1-ol : 1 p. 100 acetic acid : 1 p. 100 2-mercaptoethanol in comparison with (e) a total gluten extract (as in Fig. 5* a).

(DILL and ALSBERG, 1925). Results not reported here show that 70 p. 100 ethanol at room temperature extracts similar polypeptides to those extracted at 60 °C. Mercaptoethanol and acetic acid appear to increase the amount of protein extracted by increasing the amount of certain polypeptides, particularly the high molecular weight ones, without leading to the extraction of any novel proteins (see also Fig. 2 *e* and *f*, MIFLIN and SHEWRY, 1979). The proteins extracted by ethanol at 4 °C and propan-1-ol: acetic acid: mercaptoethanol at 60 °C were compared by 2D analysis. This separation was aimed particularly at further resolving the mass of bands of between 20 and 50 kd and the results (Fig. 3) show that there are only a few differences in this region in the polypeptides extracted by the different solvents.

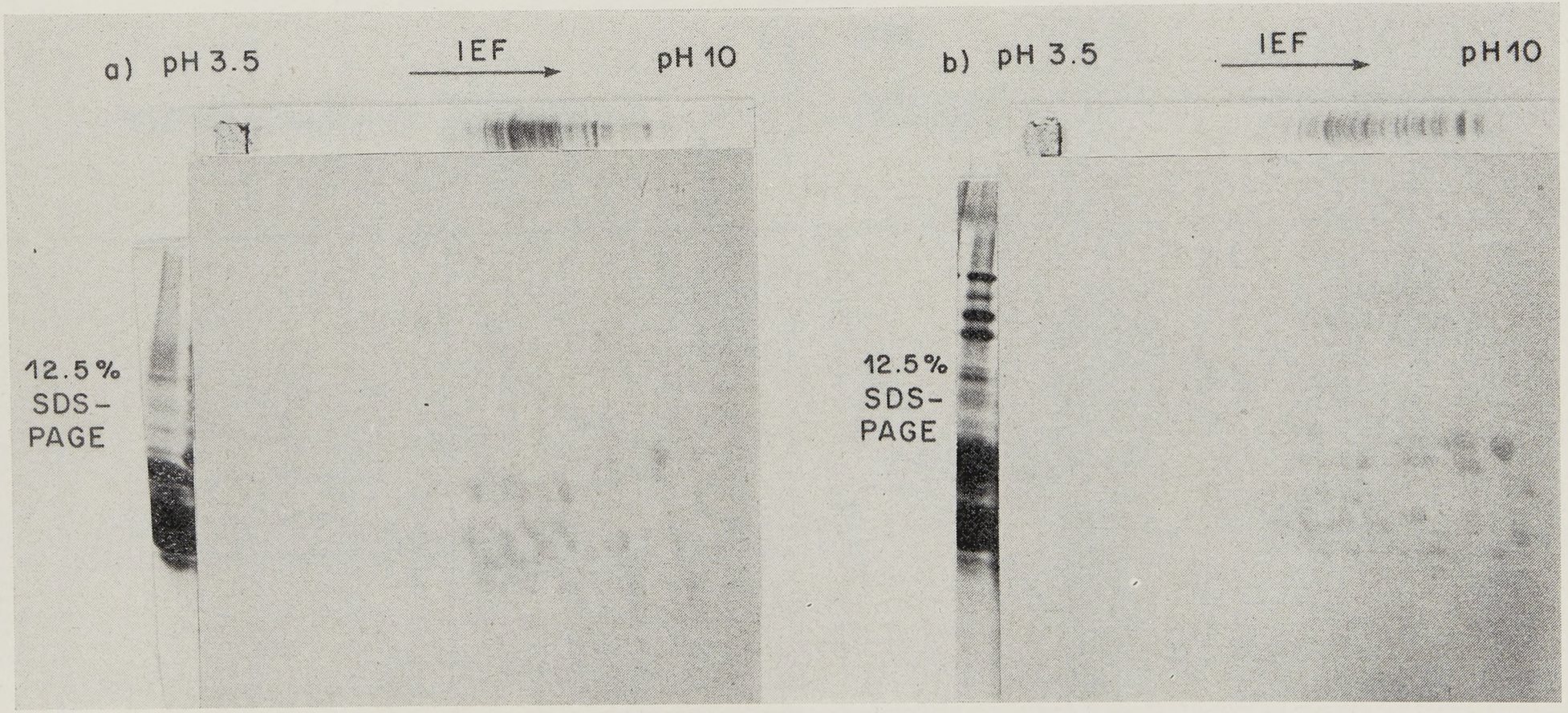

FIG. 3. — *Two dimensional separations of proteins extracted from whole milled wheat (a) 70 p. 100 ethanol at 4 °C and (b) 50 p. 100 propan-1-ol : 1 p. 100 acetic acid : 1 p. 100 2 mercaptoethanol at 60 °C. The one dimensional SDS-PAGE and IEF separations were derived from separate gels and dorol match up exactly with the 2-D separations ; they are included to provide a comparison between the two systems.*

(a)
F1
F2
(b)
(c)
F1
F2
(d)
F1
F2
Absorbance 278 nm
Elution volume
SDS-PAGE
OF

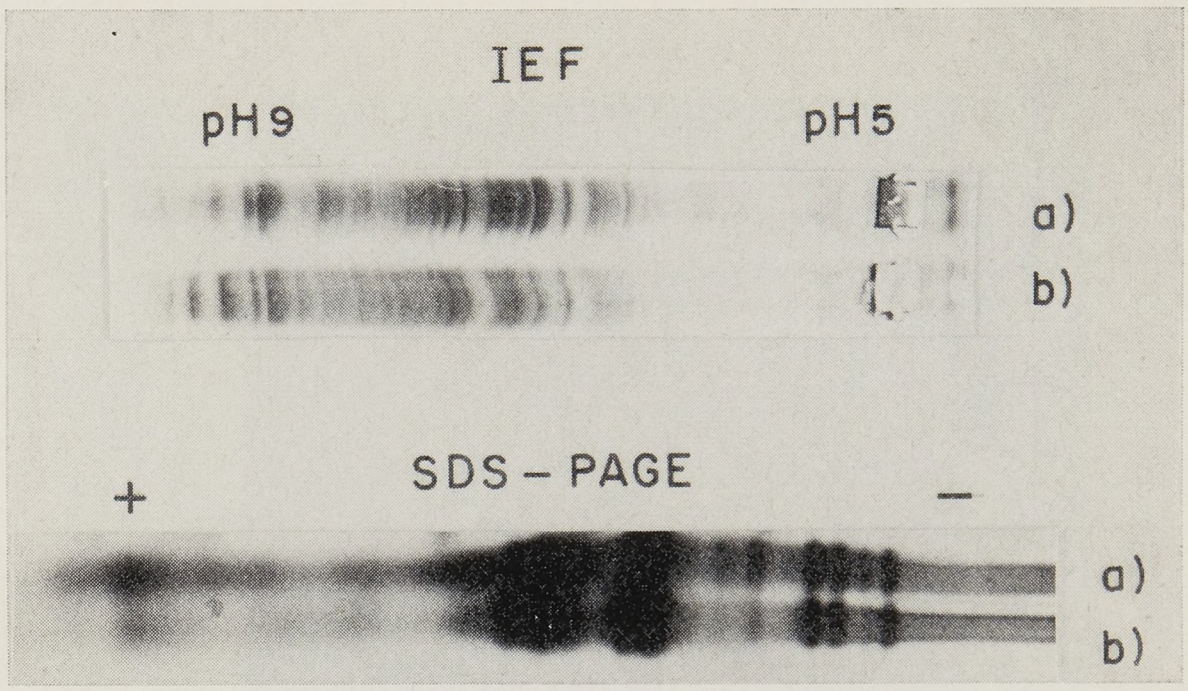

FIG. 5. — *One-dimensional IEF and SDS-PAGE of proteins extracted by urea : acetic acid : CTAB from (a) gluten and (b) protein bodies both from cv. Highbury.*

Extraction of proteins from gluten

Previous work with the AUC (0.1 M acetic acid, 3 M urea and 0.01 M CTAB) solvent of MEREDITH and WREN (1966) has given various results regarding the amount of N solubilised. Thus MEREDITH and WREN (1966) and BUSHUK and WRIGLEY (1971) solubilised about 95 p. 100 of the total flour protein whereas DANNO *et al.* (1974) and PAYNE and CORFIELD (1979) only solubilised some 70 p. 100. Our preliminary studies also indicated that only about 70 p. 100 of the total N was solublised under our conditions. However, when the concentration of urea and CTAB was increased about 98 p. 100 of the total N could be brought into solution. When this solubilised protein was chromatographed on controlled-pore glass columns two peaks of protein were eluted. The first fraction (F-1) was at the position expected of material excluded from the pores of the column whereas the second (F-2) was within the fractionating range. The size of the different protein classes are not easy to estimate using this system because of the lack of suitable markers, uncertainty regarding the amount of detergent bound per molecule and lack of information on the effect of the detergent on the conformation of the protein.

The effect of reducing agents on the distribution of gluten proteins during chromatography is shown in Figure 4 *b*. The addition of mercaptoethanol

FIG. 4. — *Gel permeation chromatography of proteins extracted by urea : CTAB : acetic acid from (a) washed gluten (cv. Copain); (b) a parallel sample to (a) but incubated with and chromatographed in the presence of 1 p. 100 2-mercaptoethanol; (c) washed gluten (cv. Highbury); (d) isolated protein bodies from developing endosperms (cv. Highbury). The SDS-PAGE gels at the side are of proteins from the stippled area of the elution profile.*

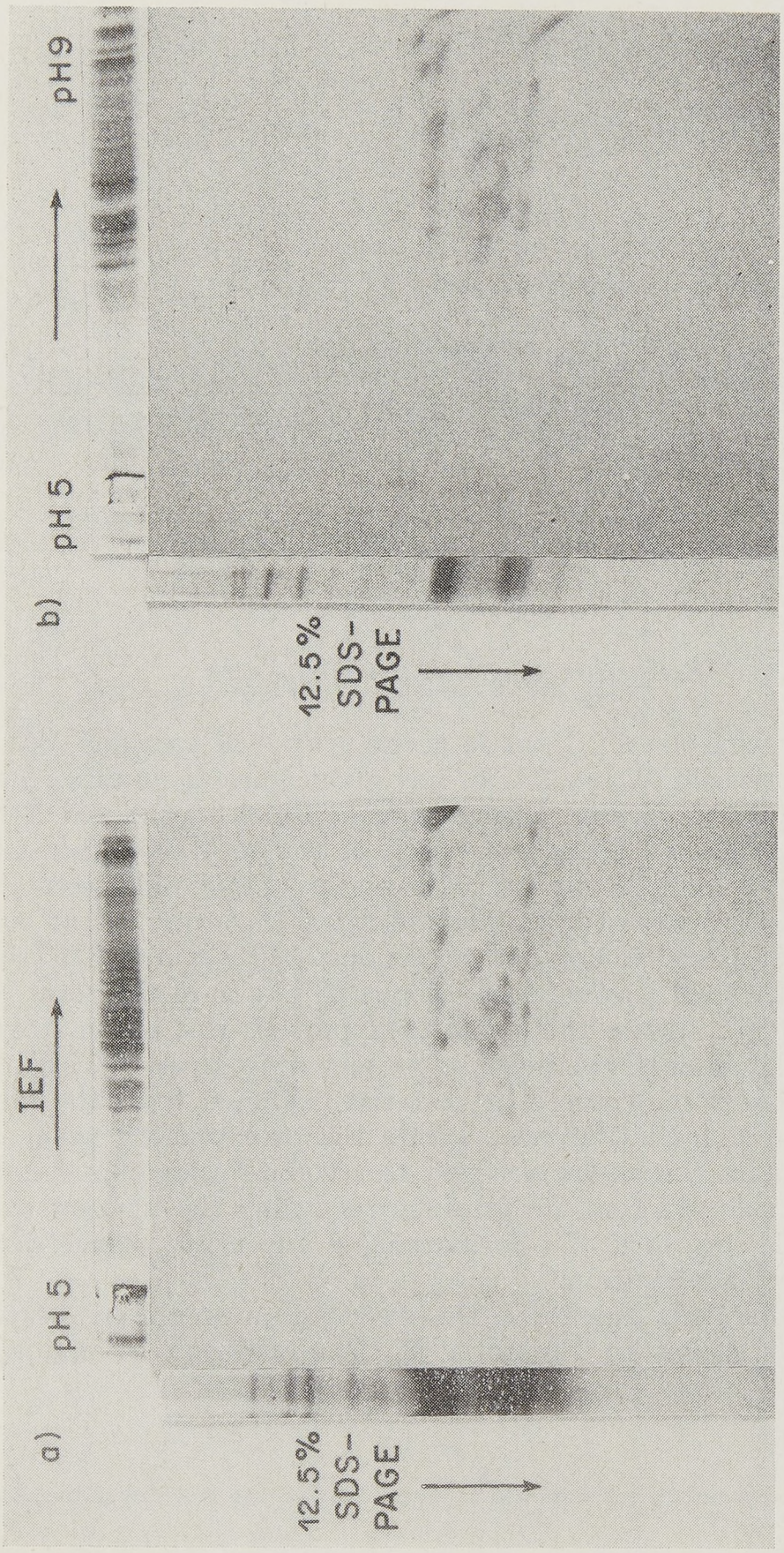

FIG. 6.A. — *Two dimensional separations of extracted wheat proteins*
Solvents: a) and b) urea: acetic acid: CTAB.
IEF pH range: a) and b) pH 5-9.
Samples: a) gluten; b) protein bodies (C. V. Highburg).

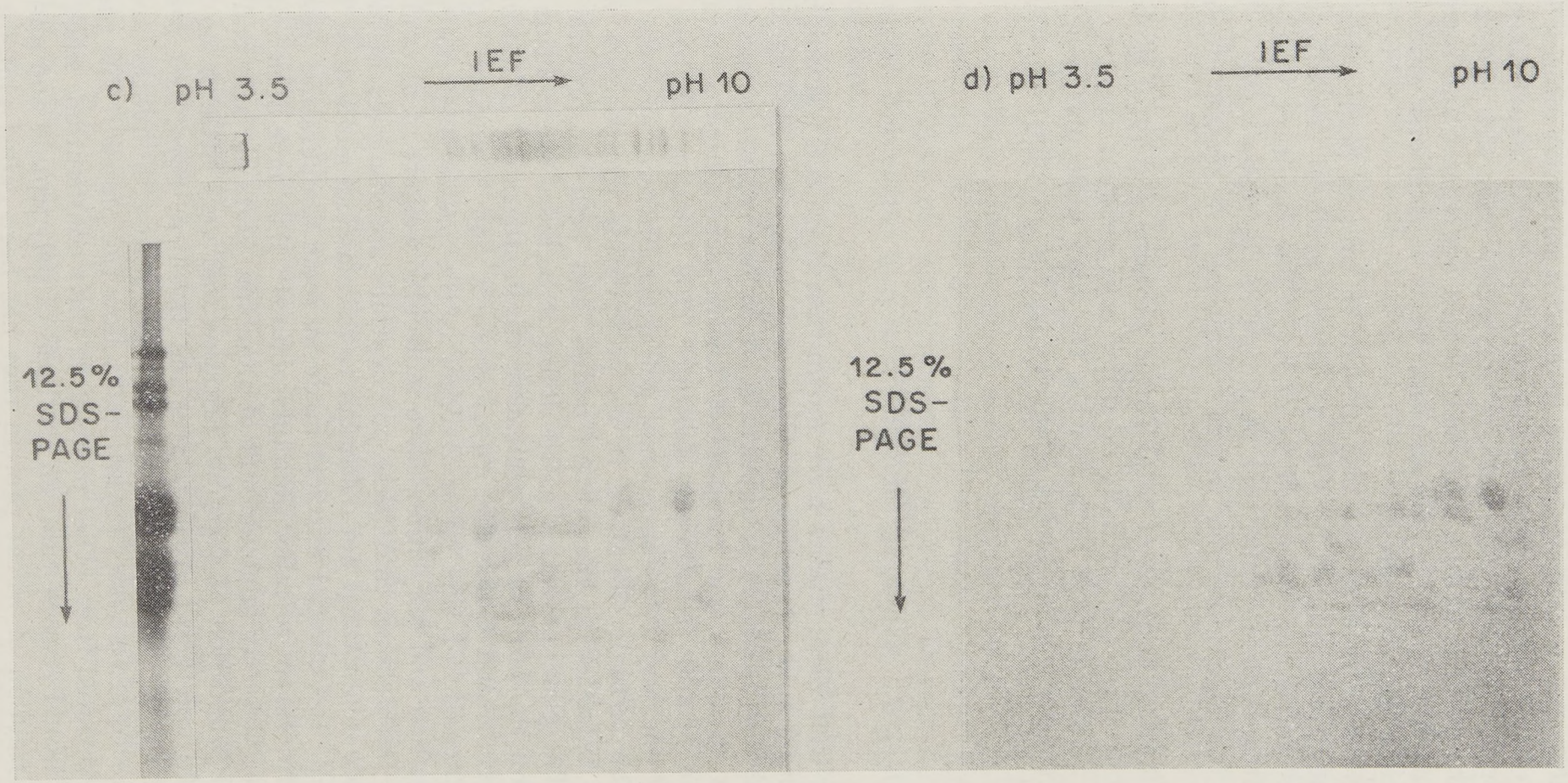

FIG. 6.B. — *Two dimensional separations of extracted wheat proteins.* Solvent: (*c*) *urea : acetic acid : CTAB ;* (*d*) *propan-1-ol : acetic acid : mercaptoethanol.* IEF pH range: (*a*) *and* (*b*) *pH 5-9,* (*c*) *and* (*d*) *pH 3.5-10.* Samples: (*c*) *protein bodies,* (*d*) *whole milled grain* (*cv. Highbury*). *The one-dimensional SDS-PAGE and IEF separations were derived from separate gels and do not match up exactly with the 2-D separations ; they are included to provide a comparison between the two systems.*

completely abolishes the high molecular weight aggregate and instead the protein elutes in a similar position to F-2.

The high molecular weight aggregate (F-1) is of potential interest since the relative amount of this fraction appears to be correlated with breadmaking quality (FIELD and MIFLIN, 1980). Other authors previously have also shown a correlation between the amount of high molecular weight aggregates and breadmaking quality (HUEBNER and WALL, 1976) although their separations were of only a portion of the total gluten protein.

To see if the F-1 fraction was also present in protein bodies during grain development, they were isolated by sucrose density gradient centrifugation and their protein extracted and separated as above. The resultant elution profile (Fig. 4 *d*) was little different from that of gluten of the same variety (Fig. 4 *c*) suggesting that the high molecular weight complexes are present during development.

Gel electrophoretic analysis of total gluten proteins and total protein body proteins show that there are virtually no differences between the two fractions (Fig. 5). It is noticeable that both contain the high molecular weight bands and have a polypeptide composition identical to the propan-1-ol: acetic acid: mercaptoethanol extract.

Discussion

We consider that these studies have shown that there is a fraction of wheat protein, representing about 60 p. 100 of the total N, which has the characteristics of gliadin in that it is insoluble in aqueous solvents but soluble in alcohol-based solvents and has an amino acid composition low in basic amino acids and high in glutamate and proline (results not shown). This fraction has been further characterised by one and two-dimensional gel electrophoretic systems and has been shown to be virtually identical in its polypeptide composition with the storage proteins deposited in the protein bodies, and with the protein fraction of the gluten. The gliadin fraction as we define it includes the fraction usually termed glutelin on the basis of gel-permeation chromatography (MEREDITH and WREN, 1966; PAYNE and CORFIELD, 1979; BUSHUK and WRIGLEY, 1971). A particular characteristic of this latter fraction is the presence of polypeptides which, after reduction and alkylation, have molecular weights of around 100 kd and above. We have shown that these polypeptides are also present in gliadin preparations (made in a manner not dissimilar to the classical methods of TELLER, 1896) and in 70 p. 100 ethanol extracts made at room temperature (results not shown). Furthermore, the so-called glutelin fractions " that have been hydrolysed with acids yield a relatively large amount of ammonia, glutamic acid and proline and a very small proportion of arginine, histidine and very little or no lysine " (e.g. see HUEBNER and WALL, 1976; KAHN and BUSHUK, 1979; and review by KASARDA *et al.*, 1977). Interestingly enough the words in quotes are taken directly from page 19 of OSBORNE (1924), and follow the phrase " The prolamins are sharply differentiated from other proteins in their constitution, since all... ". Consequently we would suggest that, on the basis of polypeptide and amino acid composition, the fraction of wheat protein that has been called glutelin on the basis of its behaviour during gel-permeation chromatography, or because of its partial extractability in 70 p. 100 ethanol, is in fact a classical prolamin and

should be called such. Further suggestive evidence that this is so is that this fraction is located within the protein bodies of the starchy endosperm; in barley and maize these bodies are composed almost entirely of prolamins (MIFLIN *et al.*, 1980).

We realise that the concept that the gliadin fraction is analogous to gluten has been proposed before. Thus O'BRIEN (1895) wrote " Previous authors consider that the constituents of gluten (gliadin and glutenin) exist in flour in the same proportions as in gluten; and that, therefore, gluten may be said to exist as such in flour. I find, on the contrary, that these substances may be extracted from gluten in varying proportions according to the method employed; this suggests one may be derived from the other and it is probably the less soluble (glutenin) that is derived from the more soluble (gliadin): hence the alcohol-soluble substance (gliadin) is ultimately coextensive with gluten. "

The gluten proteins and those deposited in the protein bodies of the developing seed are present in the form of both high molecular weight aggregates and monomeric species; the proportion of the former appears to be of some importance in breadmaking. The forces determining the aggregation are not completely understood but the maintenance of disulphide bonds are an essential component of aggregation (Fig. 3, MEREDITH and WREN, 1966; HUEBNER and WALL, 1976) and the most likely interpretation is that they are involved in cross-linking the polypeptides. We do not consider that the results obtained by KOBREHEL and COWORKERS (KOBREHEL and BUSHUK, 1977, 1978; KOBREHEL and MATIGNON, 1980) support the claims that intermolecular disulphide bonds are not important in aggregation.

If changing the name of so-called glutelin to gliadin is likely to cause confusion we would suggest that the terms " storage proteins " or " gluten proteins " are used to define the total fraction depending on whether the studies relate to the physiology of the seed or to breadmaking.

On the basis of our work we would suggest that the true glutelin fraction of wheat (originally termed glutenin by F. W. JOHNSON, see BAILEY, 1944) is not a storage protein and probably has little or no role to play in gluten formation or breadmaking. Similar concepts regarding the role of the glutelin fraction in barley and maize (but not rice) have been propounded elsewhere (MIFLIN, 1978; SHEWRY *et al.*, 1978 *b*; MIFLIN and SHEWRY, 1979; WILSON *et al.*, 1980). In these cereals it was realised a long time ago that classical OSBORNE procedures left a large proportion of the prolamin fraction behind (e.g. see BISHOP, 1939, and in the discussion to LONTIE *et al.*, 1953; LONTIE and VOETS, 1956, and SODEK and WILSON, 1971) which subsequently contaminated the glutelin fraction. This has recently been verified using one- and two-dimensional gel electrophoretic procedures (SHEWRY *et al.*, 1978 *b*; MIFLIN and SHEWRY, 1979; WILSON *et al.*, 1980). Further complications in the definition of the glutelin fraction occur when the flour is defatted prior to extraction (or subject to any other treatment causing a similar effect) leading to denaturation of albumin and globulin protein which are subsequently recovered in the glutelin fraction. The argument over gliadin and glutenin is not solely semantic but also relates to the functional similarities in the biochemistry and physiology of the endosperms of wheat, maize and barley. We believe that the understanding of cereal seed proteins is best served by a clear and consistent definition of their composition.

Reçu pour publication en octobre 1980.

Résumé

Isolement et caractérisation des protéines extraites du grain de blé entier broyé, du gluten et des corpuscules protéiques en cours de développement

Nous avons utilisé des solvants différents pour isoler les constituants protéiques de la farine de blé entière. Une délipidation préliminaire avec du butanol saturé d'eau ou avec un mélange de 2 : 2 : 1 (v/v) éthanol : diethyl éther : eau diminue la quantité d'azote extraite dans la fraction salino-soluble et augmente celle du résidu.

Après une première extraction à l'aide de 0.5 MNa Cl, le taux d'azote extrait avec des solvants alcooliques augmente en fonction de l'alcool utilisé (50 p. 100 (v/v) n-propanol > 50 p. 100 iso-propanol = 70 p. 100 éthanol), de la présence de 2-mercaptoéthanol à 1 p. 100 et en fonction de la température.

La quantité maximale d'azote (approximativement 60 p. 100 de l'azote total de la graine) a été extraite par un mélange de 50 p. 100 de n-propanol, 1 p. 100 de 2-mercaptoéthanol et de 1 p. 100 d'acide acétique à 60 p. 100. Les protéines du gluten lavées ont été étudiées après solubilisation dans le solvant acide acétique 0.01 M, urée 6 M, bromure de céthyltriméthyl ammonium 0,055 M. Ce traitement laisse moins de 5 p. 100 d'azote dans le culot après centrifugation de l'extrait à 20 000 g pendant 30 mn. Les protéines sont ensuite fractionnées par classe de taille par chromatographie sur colonnes de billes de verre poreux (diam. des pores 700 Å); on obtient deux fractions, l'une à poids moléculaire élevé et un pic plus large couvrant tout le volume de la colonne. On n'obtient pas le pic de poids moléculaire élevé lorsque les protéines sont réduites par le mercaptoéthanol. Une distribution similaire des protéines est obtenue quand on utilise les mêmes techniques pour caractériser les protéines des corpuscules protéiques de l'endosperme de blé en cours de développement.

Les différentes fractions de protéines obtenues sont comparées par électrophorèse uni- et bi-dimensionnelle. On constate que les polypeptides constituant la fraction soluble dans le solvant n-propanol, mercaptoéthanol, acide acétique du grain broyé, du gluten lavé et des corpuscules protéiques de l'endosperme en cours de développement sont essentiellement les mêmes. Nous considérons que ces polypeptides incluent la fraction prolamine des protéines de réserve des grains de blé.

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SESSION II

Gliadines du blé : structure et propriétés

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Structure and properties of α-gliadins

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Summary

Wheat varieties may be divided into two types (Type 1 and Type 2) on the basis of major features of the gel electrophoretic patterns of their α-gliadins. Almost all characterization of α-gliadins has been of components derived from Type 1 wheat varieties.

A-gliadin and (probably) related α-gliadins from Type 1 wheat varieties aggregate specifically through secondary bonding forces to form fibrillar aggregates. This aggregation seems to be dependent upon protein conformational structure; it provides a model for studying the importance of conformationally-controlled fibril formation involving other proteins in the dough system.

The A-gliadin fraction from Type 1 wheat varieties is complex and composed of many components, which results in part from coding by multiple gene copies. The genes coding for A-gliadin components may have been contributed to polyploid bread wheats (Type 1) by *Triticum urartu*. The multiplication that produced A-gliadin genes may have taken place in conjunction with speciation of *T. urartu* and its divergence from other members of the sub-tribe *Triticinae*.

A speculative model for formation of A-gliadin genes is based on the reiteration of relatively short DNA sequences (fundamental sequences) corresponding in length to only a few amino acid codons. Different fundamental sequences may have provided the basis for formation of different classes of gliadins. The reiteration of the fundamental sequence that gave rise to ω-gliadins may have taken place more recently than the reiteration that gave rise to α-, β-, and γ-gliadins.

Key words : *wheat, gliadins, composition, structure, genetics.*

I. — Introduction

The α-gliadin proteins of common bread wheat (*Triticum aestivum*) were so named by WOYCHIK *et al.* (1961) who noted that groups of bands in starch gel electrophoretic patterns of gliadins corresponded to the four major peaks of moving boundary electrophoretic patterns of gluten (JONES *et al.*, 1959) —and had the same relative mobilities in the gel— when both methods were carried out with aluminum lactate buffer, pH 3.1. Thus, the fastest-moving bands of the gel electro-

gliadins were dissolved in dilute acetic acid and dialyzed thoroughly against distilled phoretic pattern of gliadins became α-gliadins in accordance with their association with the α-gluten peak of the moving boundary electrophoretic pattern.

It has become clear over the years since 1961 that α-gliadins have many important similarities, including their compositions, to other gliadins —particularly β- and γ-gliadins— and that their study provides insight to the nature of gliadins in general. At the same time, α-gliadins have some distinctive characteristics, which may stem in part from the slightly higher proportion of basic amino acids that leads to their greater electrophoretic mobilities, and which make them of special interest.

In this paper, I plan to emphasize characterization of a particular fraction of α-gliadins that has been carried out in our laboratory. This fraction can be separated from other gliadins of certain wheat varieties by an ultracentrifugation procedure (Bernardin *et al.*, 1967); because it is not found in all wheat varieties, we have called our fraction A-gliadin to distinguish it from other α-gliadins (Platt *et al.*, 1974). I shall touch on separation and characterization of α-gliadin components carried out in other laboratories, most of which indicates a close relationship of the components to A-gliadin components. In addition, I plan to speculate on the origin and evolution of genes coding for A-gliadin components and other types of gliadins as well.

II. — Materials and methods

A. — *Wheat varieties*

Scout 66 was supplied by V. A. Johnson; *Cheyenne, Cappelle Desprez, Bezostaya,* and *Chinese Spring* were supplied by C. O. Qualset; *Marquis* was supplied by W. Bushuk; and *Justin* was supplied by R. Bieter.

The *Chinese Spring* aneuploid (nullisomic 6 A-tetrasomic 6D) was supplied originally by E. R. Sears and increased by Dr. Qualset.

B. — *Protein Fractions*

A-gliadin was prepared by the procedure of Bernardin *et al.* (1967) with some modifications (Bernardin, 1980*a*). Flour (endosperm only) was sufficiently fine to pass a 60 mesh screen. The extraction was carried out at 35 °C rather than at room temperature. Purification was carried through three ultracentrifugation steps rather than two. Column chromatography was with Bio-Gel P-60 and was carried out in 0.06 *M* ammonium formate (pH 3.1) instead of in 0.001 *M* hydrochloric acid.

Total proteins from *Scout 66* flour (endosperm only) were obtained by extraction of the flour with a mixture of 0.062 *M* Tris-HCl (pH 6.8) that included 2 p. 100 sodium dodecyl sulfate (SDS) and 5 p. 100 mercaptoethanol according to the procedure of Fullington *et al.* (1980), which involved grinding the flour and solvent together in a mortar and pestle.

Gliadins were extracted from *Scout 66* flour with 70 p. 100 (v/v) ethanol-water solution (single extraction at room temperature), the extract was clarified by centrifugation, and the extracted gliadins precipitated from the supernatant by a three-fold dilution with 1.5 p. 100 sodium chloride solution. The precipitated

water. The supernatant was clarified by centrifugation to remove slight turbidity and the clear supernatant was freeze-dried as the gliadin preparation.

Glutenins consisted of the residue remaining after the extraction of *Scout 66* flour with ethanol-water solution. This residue included the starch, but presumably the proteins present were equivalent to glutenins.

C. — *Methods*

Polyacrylamide gel electrophoresis (PAGE) in one dimension was carried out in aluminium lactate buffer (pH 3.2) as described by KASARDA *et al.* (1976*b*) except that proteins were extracted from de-germed seeds or flour with 1.5 *M* dimethylformamide (DMF) containing 20 p. 100 sucrose.

Two-dimensional PAGE, which consisted of migration in the first dimension in aluminium lactate buffer at pH 3.2 followed by equilibration of the gel with a Tris-glycine-DMF buffer at pH 9.2 and electrophoresis in the second dimension, was carried out as described by MECHAM *et al.* (1978).

SDS-PAGE was carried out according to the method of PAYNE and CORFIELD (1979).

Transmission electron microscopy was carried out as described by KASARDA *et al.* (1967) and BERNARDIN and KASARDA (1973*a*; 1973*b*). Samples were shadowed with uranium to produce contrast.

Fractionation of A-gliadin components on diethylaminoethyl (DEAE)—cellulose (DE-32) was carried out according to procedures of BERNARDIN and DIETLER (1980). Their fractionation was carried out in 4 *M* urea with a gradient of ammonium acetate ranging from zero to 0.01 *M* at pH 6.

Amino acid sequencing was carried out as described by KASARDA *et al.* (1974) and AUTRAN *et al.* (1979).

Circular dichroism measurements were carried out as described by KASARDA *et al.* (1968).

III. — Results and discussion

A. — *Characterization of α-Gliadins*

a) *Varietal differences and chromosome associations*

Figure 1 shows one-dimensional PAGE patterns of eight different wheat varieties, A-gliadin prepared only by ultracentrifugation without further purification by chromatography, and the pattern of an aneuploid derivative of *Chinese Spring* that is missing chromosome 6A and has compensating extra doses of chromosome 6D (nulli 6A-tetra 6D). Differences may be noted in the patterns of α-gliadins in the different varieties, but most varieties can be divided on the basis of their α-gliadin patterns into two major types. For convenience, I shall refer to these divisions as Type 1 and Type 2.

Cappelle Desprez, INIA 66, Cheyenne, Scout 66, and *Bezostaya* belong to Type 1, which has a pattern similar to that of A-gliadin, whereas *Chinese Spring, Justin,* and *Marquis* belong to Type 2, which does not include an A-gliadin-like pattern, but which has two fast-moving bands that can be seen clearly as the two

fastest-moving bands of *Chinese Spring* in figure 1. The A-gliadin of figure 1 was prepared from *Scout 66*, but probably all Type 1 varieties would yield A-gliadin by the centrifugation procedure of BERNARDIN *et al.* (1967) although only a few have been tested (PLATT, *et al.*, 1974). It is our general impression based on PAGE patterns that Type 1 wheats have more protein corresponding to α-gliadins than Type 2 wheats. The genes coding for A-gliadin components may be alleles of the genes coding for the two fastest-moving components in the pattern of Type 2 wheats (MECHAM *et al.*, 1978).

A-gliadin components are coded by genes located on chromosome 6A (KASARDA *et al.*, 1976*b*). Other α-gliadins are coded by genes on chromosomes

FIG. 1. — *PAGE patterns of gliadins extracted from different wheat varieties compared with the pattern of A-gliadin (e) that had been prepared only by ultracentrifugation (3X) and gliadins of an aneuploid derivative of* Chinese Spring *(h), which was missing chromosome 6A, but which was compensated by two extra doses of chromosome 6D.*

6A or 6D (SHEPHERD, 1968; WRIGLEY and SHEPHERD, 1973; MITROFANOVA, 1976; KASARDA *et al.*, 1977). It can be seen in the gel patterns of Figure 1 that the absence of chromosome 6A in *Chinese Spring* results in the loss of several α-gliadin components-including the two fastest-moving components; the remaining α-gliadins are coded by genes on chromosome 6D. Although A-gliadin makes up most of the α-gliadin of Type 1 wheats, some components are not collected in the pellet during the ultracentrifugation procedure and it is not known which chromosome controls the synthesis of these non-aggregating α-gliadins.

A-gliadin appears mainly as two components in one-dimensional PAGE (fig. 1), but 6 to 8 components are revealed by two-dimensional electrophoresis (fig. 2).

The two-dimensional pattern of A-gliadin is evident in the pattern of *Cheyenne* gliadins as shown in figure 3. MECHAM and KASARDA (1980) have noted a similarity in patterns of A-gliadin components and a group of α-gliadins observed in some accessions of *Triticum urartu;* the pattern of gliadins from one of these accessions is shown in figure 4. *T. urartu* has been suggested as the donor of the A genome to polyploid wheats of the AB genome type (KONAREV *et al.*, 1979; CHAPMAN *et al.*, 1976) and the similarity in pattern we have observed for A-gliadin components and α-gliadins of *T. urartu* supports the possibility that *T. urartu* contributed the A genome to Type 1 wheats.

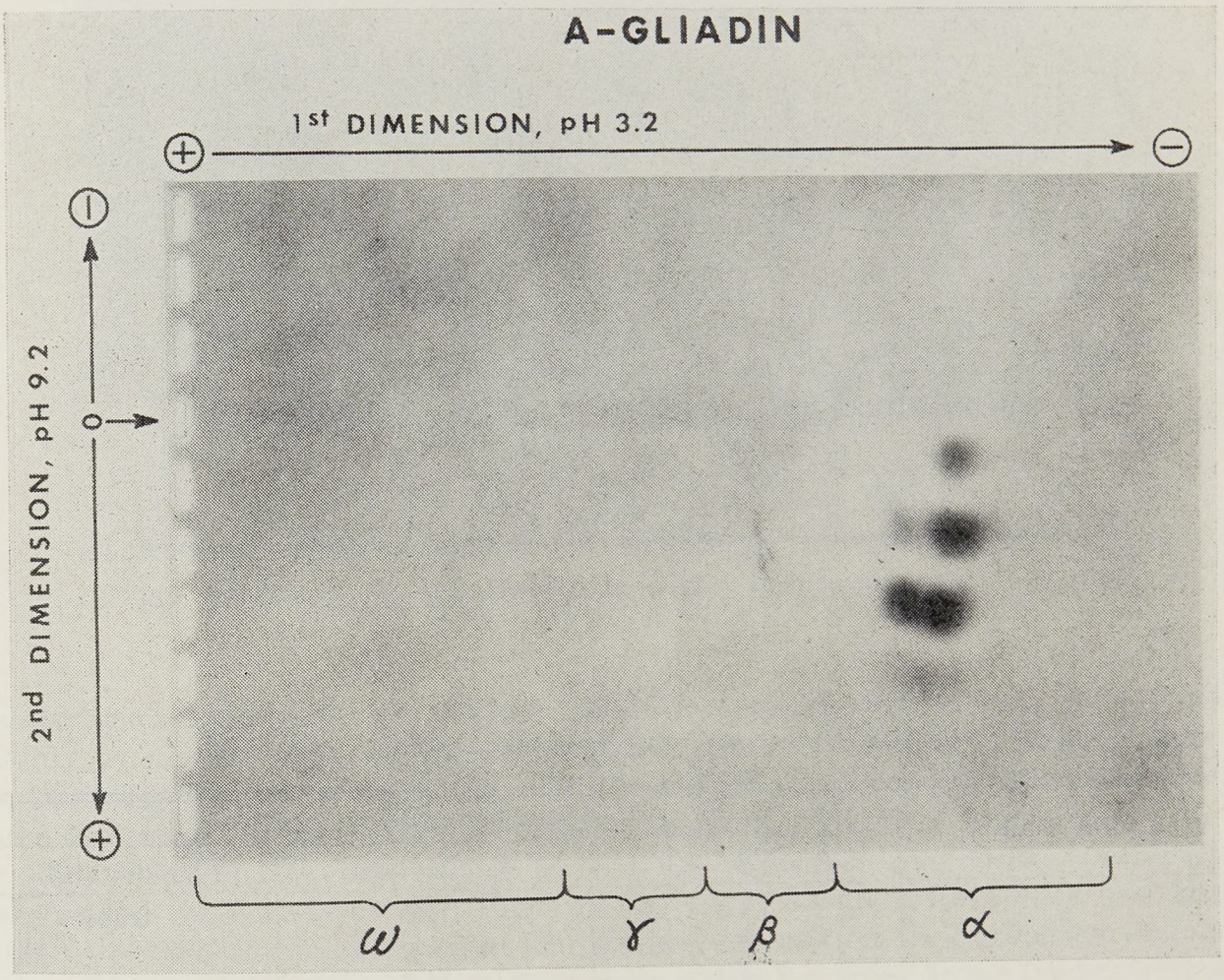

FIG. 2. — *Two-dimensional PAGE of A-gliadin.*

The A genome to Type 2 wheats must have been contributed by (or undergone introgression by) another quite different type of *T. urartu* or, alternatively, it was contributed by *T. boeoticum,* which is also a candidate for progenitor of the A genome to polyploid wheats.

b) *Relationship of A-gliadins and other α-gliadins*

The amino acid compositions of two A-gliadin components are compared with the composition of a γ_1-gliadin (HUEBNER *et al.*, 1967) in Table 1. The compositions of all three components are strikingly similar. The only significant differences between the A-gliadin components are one additional lysine in one and

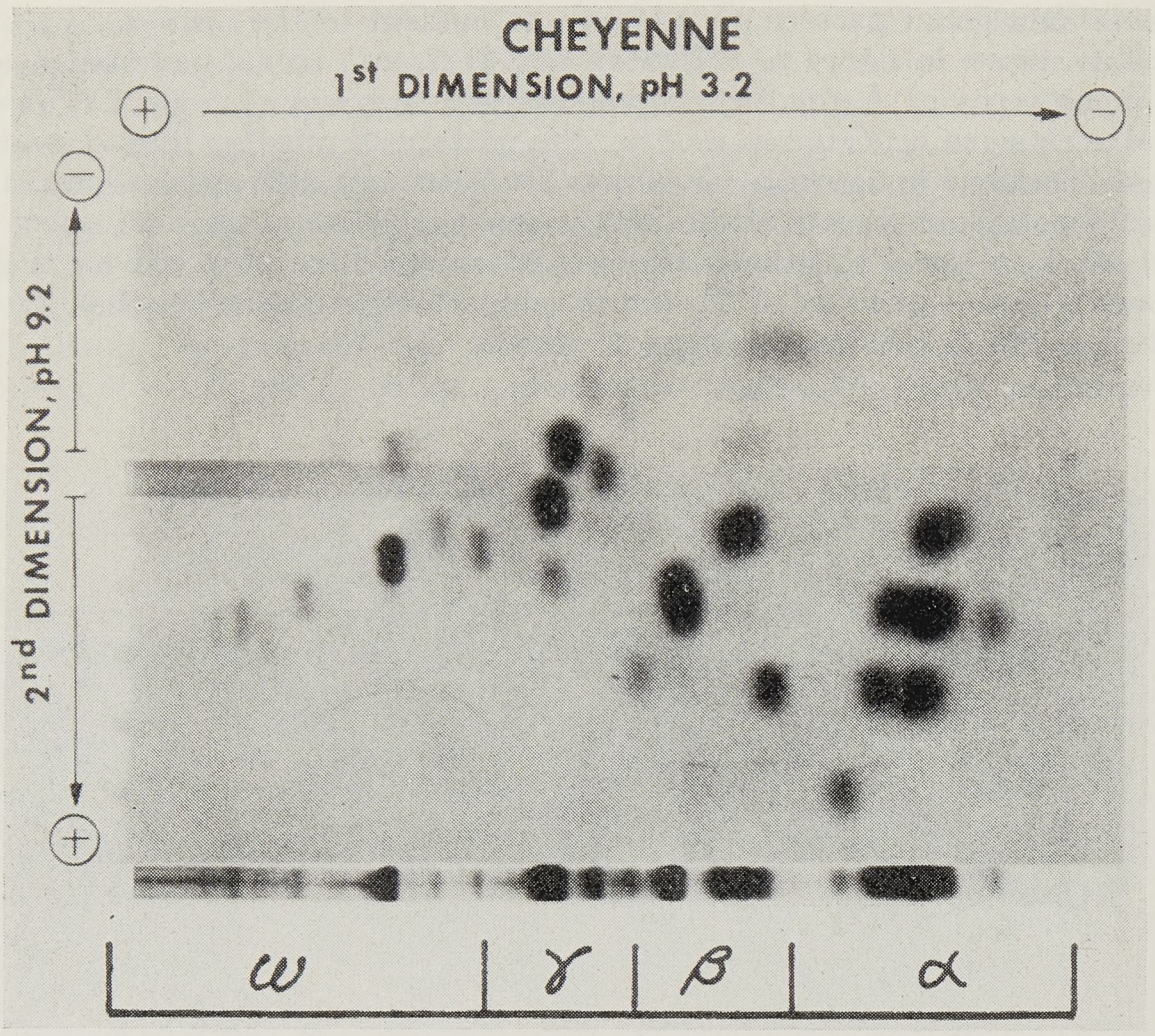

FIG. 3. — *Two-dimensional PAGE of gliadins from the variety* Cheyenne.

TABLE 1

Amino acid compositions of A-gliadin fractions and of γ_1*-gliadin*

Amino Acid	Residues per mole (32,000)		
	A_{1B}-gliadin[1]	A_2-gliadin[1]	γ_1-gliadin[2]
Lys	1.7	1.0	trace
His	5.5	5.6	4.2
Arg	5.1	4.7	4.0
Asp	7.7	8.0	8.0
Thr	4.1	4.0	5.4
Ser	15.0	14.8	15.0
Glu	105	108	111
Pro	40.0	40.6	42.6
Gly	6.8	6.9	6.8
Ala	7.0	7.4	9.4
Cys/2	6.0	5.2	5.8
Val	12.9	12.3	12.2
Met	2.0	2.8	2.2
Ile	12.1	12.3	13.0
Leu	23.1	22.1	19.8
Tyr	8.6	8.5	7.8
Phe	9.8	9.8	10.2
Trp	1.1	1.1	0.6

(1) PLATT and KASARDA, 1971.
(2) HUEBNER *et al.*, 1967.

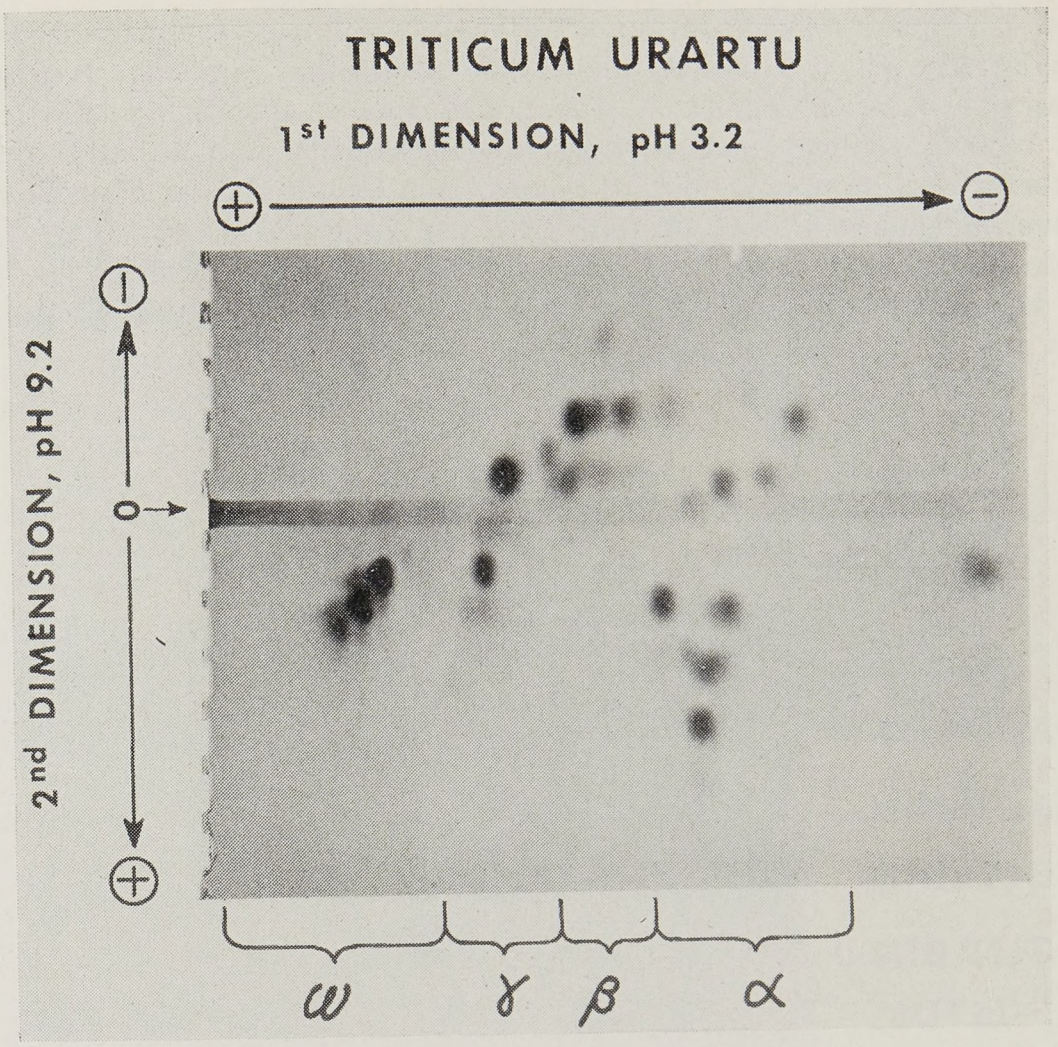

FIG. 4. — *Two-dimensional PAGE of gliadins from* Triticum urartu.

one additional methionine in the other and the γ_1-gliadin differs from the A-gliadins mainly in having three or four fewer basic groups, which is in accord with its considerably lesser electrophoretic mobility at pH 3. This γ_1-gliadin also has the same N-terminal amino acid sequence as A-gliadin (BIETZ *et al.*, 1974).

Other α-gliadin components have been separated and their compositions determined by EWART (1976), BIETZ *et al.* (1977), and CHARBONNIER and MOSSE (1980). These components were similar in composition to A-gliadins and, in a sense, may be A-gliadins in that they were separated from Type 1 wheats: *Ponca* (BIETZ *et al.*, 1977); *Cappelle Desprez* (EWART, 1976); *Cappelle* (CHARBONNIER and MOSSÉ, 1980). *Ponca* definitely contains A-gliadin (PLATT *et al.*, 1974) and I suspect that *Cappelle* and *Cappelle Desprez* would also yield A-gliadin if tested by the ultracentrifugation procedure. To the best of my knowledge, no α-gliadins have been separated and characterized from Type 2 wheat varieties and we cannot say what differences there might be in these components, which are coded by genes of chromosomes 6A and 6D, as compared with A-gliadins, which are coded by genes of chromosome 6A.

c) *Molecular weight and aggregation into fibrils*

Figure 5 shows SDS-PAGE patterns of A-gliadins, the gliadin mixture, and glutenins from *Scout 66*. The A-gliadin resolved into two heavy bands with molecular weights (MW's) of 29 and 31 $\times$ 10^3 and a third, fainter band with

MW 27 × 10^3 based on calibration of this gel. A lower-resolution approach to SDS-PAGE had yielded a slightly-higher MW and failed to resolve A-gliadin into more than one band (PLATT *et al.*, 1974; KASARDA *et al.*, 1976*a*); the resolution achieved by this present method does not necessarily indicate different MW's for the resolved components in that it has been demonstrated that SDS-PAGE can resolve, in some instances, proteins with the same MW's but slightly different compositions (DE JONG *et al.*, 1978; NOEL *et al.*, 1979). A MW of 30 × 10^3, as found in this instance, is close to the MW of 31 × 10^3 estimated by PLATT and KASARDA (1971) from amino acid composition data.

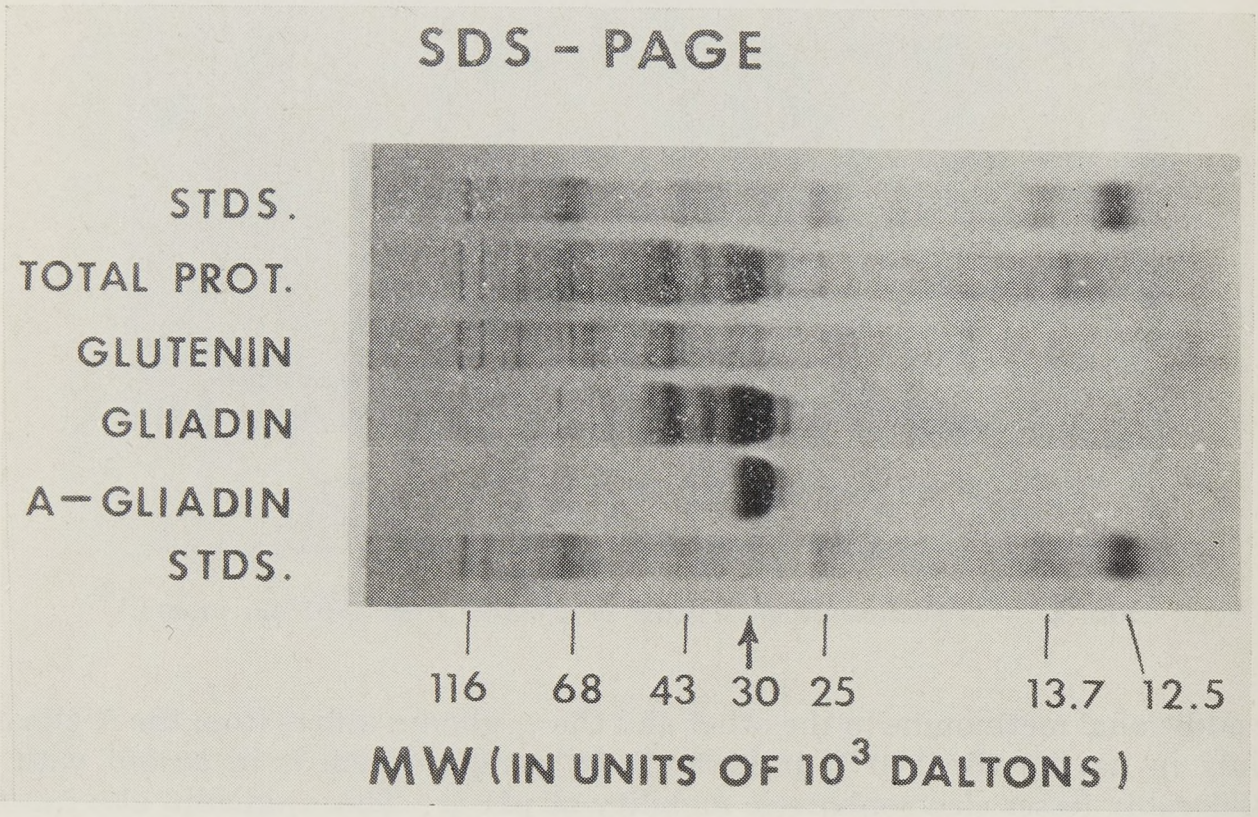

FIG. 5. — *SDS-PAGE of proteins extracted from the variety Scout 66 : a, total proteins; b, glutenins; c, alcohol-soluble gliadins; d, A-gliadin; and e, standard proteins* (β-*galactosidase, 116* × 10^3; *bovine serum albumin, 68* × 10^3; *ovalbumin, 43* × 10^3; *chymotrypsinogen A, 25* × 10^3; *ribonuclease, 13.7* × 10^3; *and horse-heart cytochrome C, 12* × 10^3). *Migration was from left to right.*

The ultracentrifugation procedure for the separation of A-gliadin from other proteins of Type 1 wheat varieties (BERNARDIN *et al.*, 1967) works because the A-gliadin protein molecules aggregate preferentially into fibrillar forms (KASARDA *et al.*, 1967). In this procedure, proteins are extracted from flour with 0.01 *M* acetic acid (ratio of acid to flour, 4:1) and the final pH of the extracted mixture is close to pH 5. Under these conditions, the A-gliadin is aggregated and, after the extract is clarified by low-speed centrifugation, can be collected by ultracentrifugation. The fibrillar nature of the protein collected at this first ultracentrifugation can be seen in the electron micrograph of figure 6. The appearance of the specimen gives the impression of a paper-like mat of fibrils imbedded in a film of some sort-possibly unaggregated protein or aggregated protein that is not fibrillar in nature.

Aggregated A-gliadin that has been highly purified is shown in figure 7. This

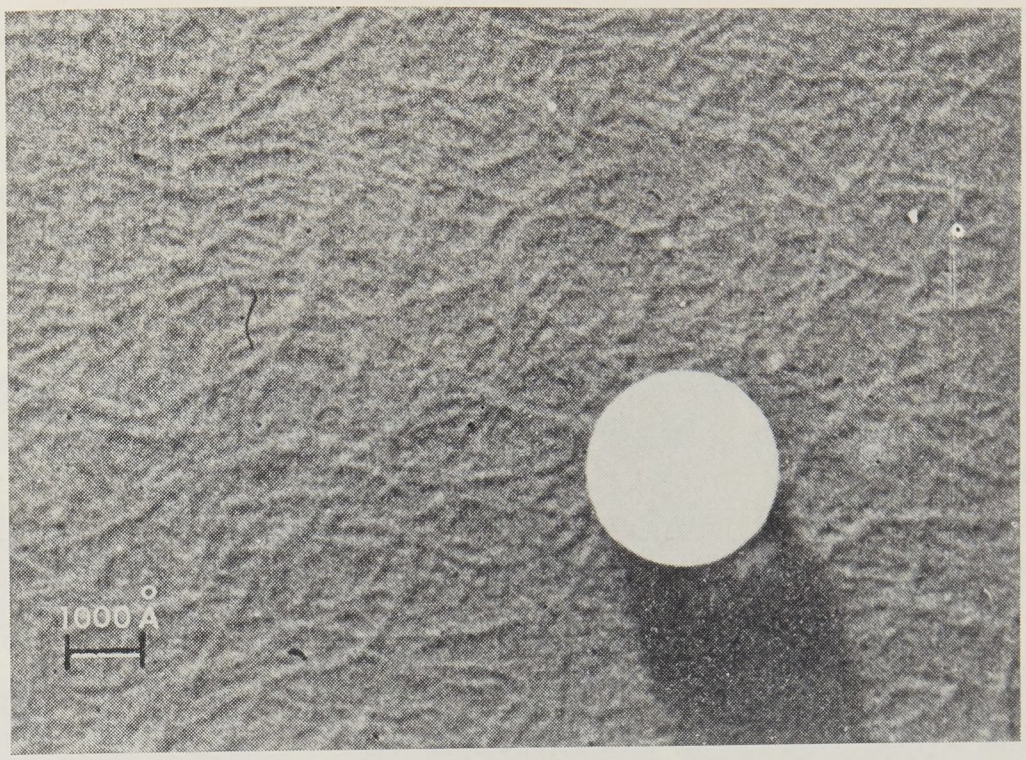

FIG. 6. — *Transmission electron micrograph of A-gliadin fibrils obtained from the first ultracentrifugation of an acetic acid (0.01 M) extract of wheat flour (from Scout 66). Uranium shadowed; polystyrene bead provides a reference length of about 0.264 μ.*

micrograph shows that aggregated A-gliadin consists of uniform fundamental fibrils that can aggregate laterally to form bundles of fibrils, which are interconnected by common fundamental fibrils. The uniformity of the fundamental fibrils of A-gliadin suggests that the interacting subunits have fixed, stable confor-

FIG. 7. — *Transmission electron micrograph of A-gliadin fibrils obtained from a purified A-gliadin preparation. Uranium shadowed; polystyrene bead provides a reference length of about 0.264 μ.*

mations rather than flexible, random coil conformations. Recent X-ray diffraction studies of oriented A-gliadin fibrils (BERNARDIN, 1980*b*) indicate that the diameter of the fundamental fibril is about 60 Å rather than the 80 Å we had estimated earlier (KASARDA *et al.*, 1967) on the basis of electron micrographs. Either diameter would be about twice as large, however, as the diameter we would expect for an approximately spherical, compactly-folded protein molecule with MW equivalent to that of the A-gliadin subunit. The organization of subunits in the

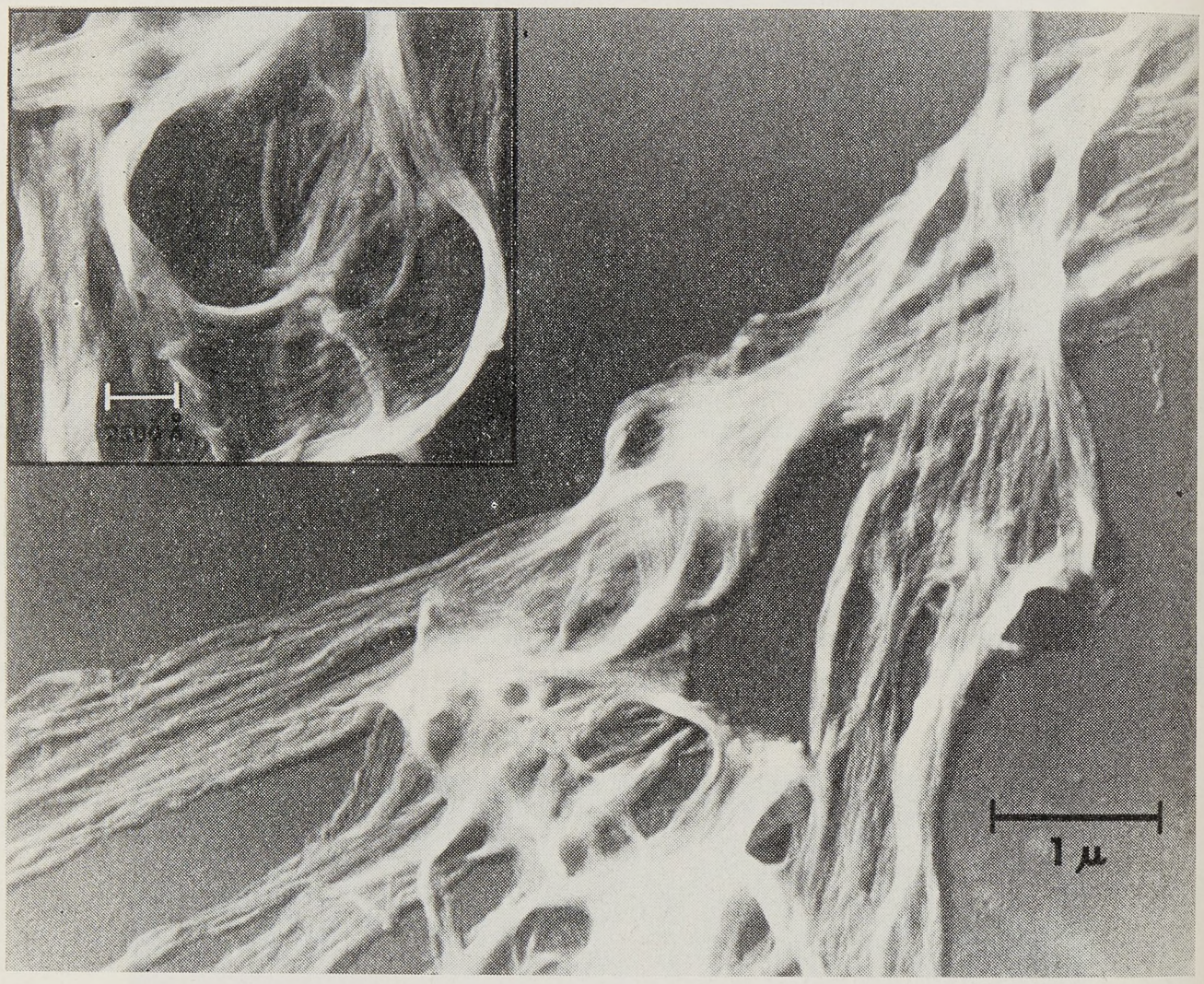

FIG. 8. — *Transmission electron micrograph of fibrils formed by wetting of flour particle; from* BERNARDIN *and* KASARDA (1973*b*).

fibrils may be more complex than that of a simple string of beads. Another possibility, however, is that the subunit may not be spherical in shape —it could, for example, be flattened like a disk although we do not think this is likely.

BERNARDIN and KASARDA (1973*a*; 1973*b*) reported that wetting of flour or endosperm particles of wheat instantly produced networks of fibrils that were pulled away from endosperm cells with broken cell walls. These fibrils were visible in the light microscope, but when they were examined by transmission electron microscopy, the larger fibrils visible in the light microscope were revealed to consist of finer fibrils, the finest of which approached in diameter the fundamental A-gliadin fibrils as is illustrated in figure 8. These endosperm fibrils were formed by all wheats (undamaged by heat), including Type-2 varieties. We think that most wheat storage proteins participate in this fibril formation and

suspect that these fibrils contribute importantly to the viscoelastic properties of doughs.

The formation of fibrils from A-gliadin subunits involves only secondary bonding forces—hydrogen bonds, hydrophobic bonds, ionic bonds. No disulfide or other primary chemical bonds participate in the aggregation, which is readily reversed by decreasing solution pH or by increasing the temperature beyond about 30 °C (KASARDA and PLATT, 1980). The aggregation of A-gliadin provides a useful model for study of protein-protein interactions in doughs (BERNARDIN, 1975, 1978). Although other factors such as intermolecular disulfide bonding may play a role in determining dough properties—our knowledge of the system is far from complete—I think it likely that specific secondary bonding closely related to protein conformation also plays an important role.

d) *Subunit conformation*

Because of our interest in the nature of subunit interactions important for fibril formation and the role of protein conformation in determining these interactions, we have studied the conformational structure of A-gliadin by means of circular dichroism (CD), the differential absorption of right and left circularly pola-

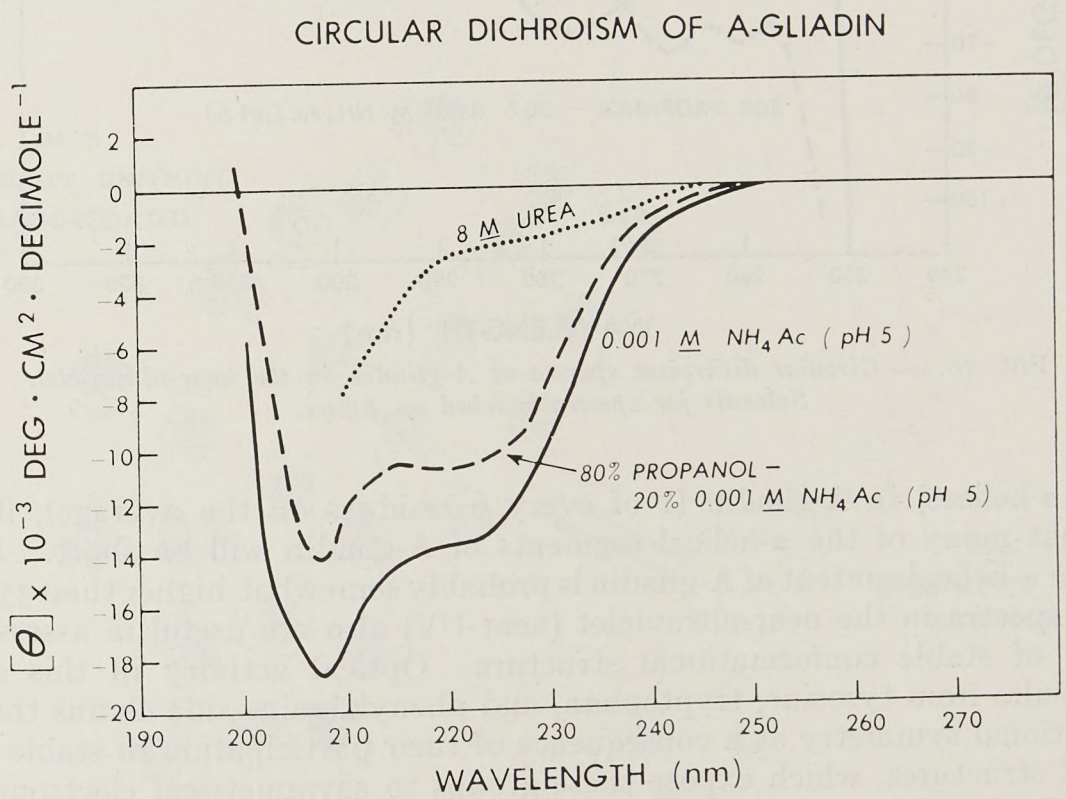

FIG. 9. — *Circular dichroism spectra of A-gliadin in the far-ultraviolet. Solvents for spectra marked on figure.*

rized light by optically active molecules. CD spectra in the far-ultraviolet (far-UV) ranges of wavelength have provided information about the conformational structure of the polypeptide chain of A-gliadin. The CD spectrum of A-gliadin in 0.001 *M* ammonium acetate (pH-5) shown in figure 9 indicates, on the basis of negative CD bands near 222 and 208 nm, that A-gliadin has a significant amount

of α-helical structure; this is in accord with our earlier work (KASARDA *et al.*, 1968). Comparison of the optical activity (elipticity [θ]) of the 222 nm CD band with that found for synthetic polyamino acids in the α-helical form indicates that A-gliadin has about 35 p. 100 of its polypeptide chain in the α-helical conformation.

The optical activity of α-helices is dependent, however, on their length, or the number of amino acids involved in each stretch of helix. CHEN *et al.* (1974) concluded, for example, that α-helices with 9 amino acid residues would contribute only about 75 p. 100 as much optical activity at 222 nm as α-helices with 40 residues. Because of the large numbers of proline residues (which cannot partici-

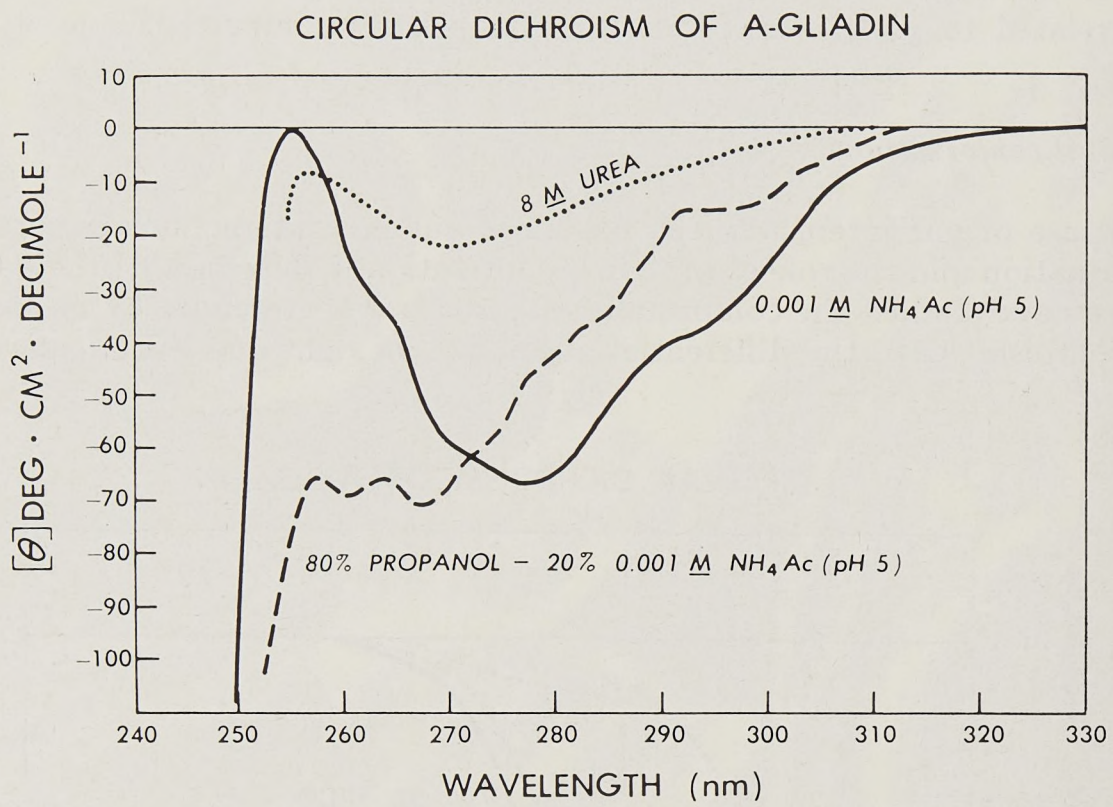

FIG. 10. — *Circular dichroism spectra of A-gliadin in the near-ultraviolet. Solvents for spectra marked on figure.*

pate in α-helices) in A-gliadin (1 of every 6 residues on the average), it seems likely that many of the α-helical segments of A-gliadin will be short. Accordingly, the α-helical content of A-gliadin is probably somewhat higher than 35 p. 100.

CD spectra in the near-ultraviolet (near-UV) also are useful in assessing the presence of stable conformational structure. Optical activity in this spectral range results from tyrosine, tryptophan, and phenylalanine side chains that have lost rotational symmetry as a consequence of their participation in stable conformational structures, which expose these groups to asymmetrical electromagnetic fields. Cystine side chains might also contribute to optical activity in the near-UV as a consequence of inherent asymmetries. The near-UV CD spectrum of A-gliadin in 0.001 *M* ammonium acetate (pH 5) is shown in figure 10. The presence of significant optical activity near 280 nm for A-gliadin indicates that the single tryptophan residue and one or more tyrosines are involved in fixed conformational structure. This provides strong support for a compactly-folded conformation in A-gliadin. Loss of this activity at pH 3 (KASARDA *et al.*, 1968) accompanied by a small decrease in the amount of α-helix in A-gliadin suggests that the protein undergoes a partial unfolding at acid pH. A strongly dissociating solvent,

such as 8 *M* urea, largely destroys optical activity in the near-UV for A-gliadiu and causes its CD spectrum in the far-UV to become characteristic of a protein with a flexible, random coil conformation.

Figures 9 and 10 also show CD spectra for A-gliadin in an 80 p. 100 n-propanol 20 p. 100 0.001 *M* ammonium acetate solution. The change in the near-UV spectrum indicates that a conformational change occurs in the A-gliadin molecule in going from aqueous solution to alcohol-water solution although the optical activity indicates that the new conformation is a stable one. The optical activity characteristic of tyrosine and tryptophan side chains has diminished and new bands near 260 and 268 nm may result from stable structures involving phenylalanine side chains, insofar as similar bands have been observed in a protein that has no tyrosine or tryptophan (Luer and Wong, 1979). The CD spectrum in the far-UV of A-gliadin in propanol solution indicates a 20 p. 100 decrease in α-helical content of the protein as compared with aqueous solution in 0.001 *M* ammonium acetate.

e) *Schematic representation of A-gliadin aggregation*

Our understanding of A-gliadin aggregation in terms of subunit conformation is represented schematically in figure 11. At pH 3, A-gliadin is dissociated and partially unfolded as a consequence of excess positive charge. As the pH is

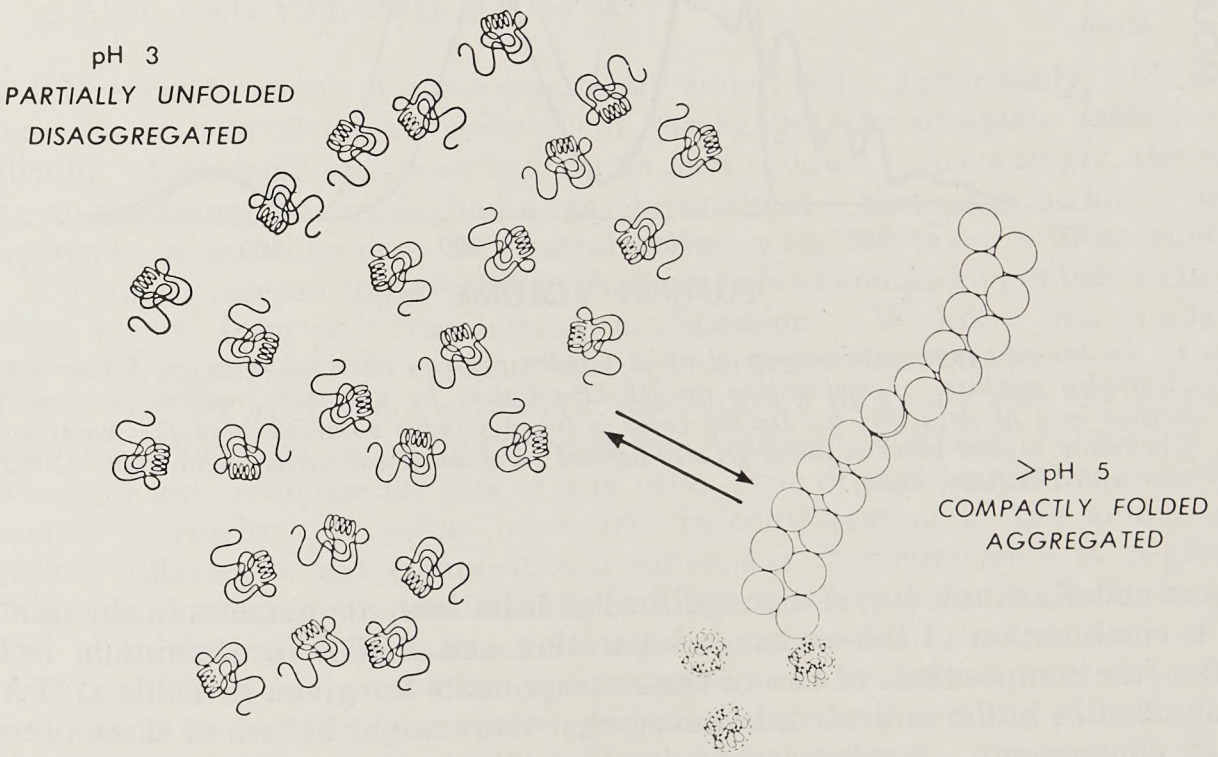

Fig. 11. — *Schematic representation of the aggregation of A-gliadin protein subunits into fibrillar forms.*

increased, free carboxyl groups ionize, which decreases the net charge of the molecule. The subunits become compactly folded and aggregate in a specific way to form microfibrils; this aggregation is enhanced by increased ionic strength. At pH 5 and salt concentrations of 0.005 *M*, the MW of the aggregates is several

million and the MW continues to increase as pH and ionic strength are increased further. The microfibrils formed can aggregate in lateral fashion to form an interconnected network.

f) *Multiplicity of A-gliadin components: Post-translational modification or multiple genes?*

A-gliadin, as defined by its aggregation and collection by way of our ultracentrifugation procedure (Bernardin *et al.*, 1967), is composed of a number of clasely similar protein components. The number of components that can be distinguished in A-gliadin is dependent upon the methods used to define them.

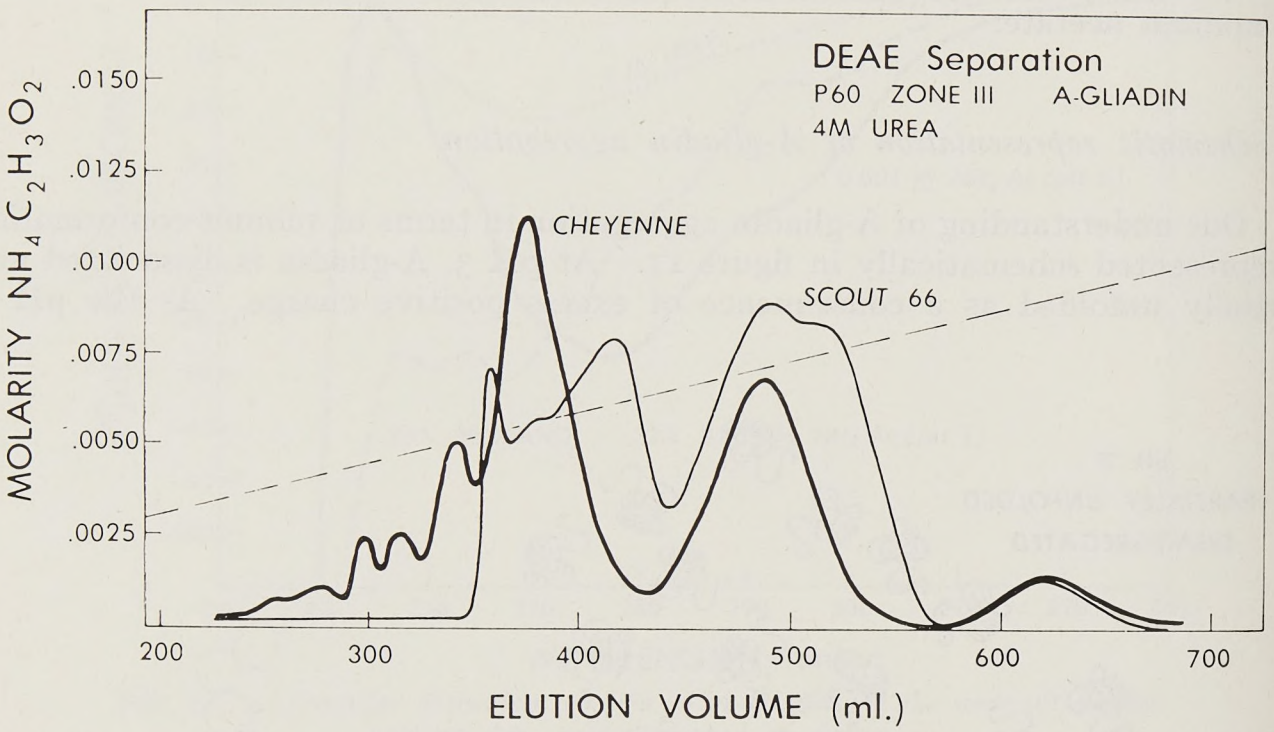

Fig. 12. — *Ion-exchange fractionation of an A-gliadin fraction obtained from gel filtration of the A-gliadin mixture. Fractionation on DEAE-cellulose by a linear gradient of ammonium acetate in 4 M urea, pH 6. Darker curve is for A-gliadin obtained from the wheat variety* Cheyenne; *lighter curve is for A-gliadin derived from the wheat variety* Scout 66. (Bernardin *and* Dietler, *1980*).

Platt and Kasarda (1971) distinguished at least four components in the mixture by a combination of ion-exchange separation and PAGE in aluminium lactate buffer (the compositions of two of these components are given in Table 1); PAGE in an alkaline buffer indicated, however, that there might be two or three times as many components. Isoelectric focusing of A-gliadin (Autran *et al.*, 1980*a*) yields patterns with 12 to 16 bands although only three or four bands are major and the others vary in intensity from weak to moderate.

Recently, Bernardin and Dietler (1980) have studied the complexity of the A-gliadin fraction by combining gel filtration chromatography, ion-exchange chromatography on several different media and at several different pH's, and PAGE at several different pH's. Their comparison of a gel filtration fraction of A-gliadins from two different wheat varieties by ion-exchange chromatography

on DEAE-cellulose at pH 6 is shown in figure 12. The patterns show a number of peaks —and differences between the fractions derived from two different wheat varieties. Refractionation of material derived from a single peak of the DEAE-cellulose fractionation on a different ion-exchange medium (carboxymethyl-cellulose) usually resolved several more peaks. They concluded that their fractionations provided evidence for 24 components in A-gliadin. These separations result mainly from differences in charged side chains. Lest these results seem in conflict with my earlier contention that A-gliadin components are highly similar, I point out that 24 components in the mixture could result from random combination of only 5 differences in charged side chains —such as a free carboxyl group in contrast to an amidated form, an additional lysine, one less histidine, and so forth.

But, in addition to differences in charged side chains, the components of A-gliadin also differ in some neutral amino acids. The A_2-component has one more methionine residue than the A_{1B}-component (Table I) and, although A-gliadin and α-gliadins have one major N-terminal amino acid sequence (KASARDA *et al.*, 1974; BIETZ *et al.*, 1977) that is characteristic of more than half of all gliadins of *Scout 66* (AUTRAN *et al.*, 1979), we have found evidence for minor components that differ in this sequence for certain of the neutral amino acids. The first six residues of the major sequence are VAL-ARG-VAL-PRO-VAL-PRO-, but we have evidence for the following additional N-terminal sequences in the A-gliadin mixture:

(1) *ALA*-ARG-VAL-PRO-VAL-PRO-
(2) VAL-ARG-*PHE*-PRO-VAL-PRO-
(3) VAL-ARG-VAL-PRO-*MET*-PRO-.

The differences from the major sequence are underlined. Presumably, additional differences in neutral amino acids occur throughout the complete sequence of A-gliadin, which includes about 275 amino acid residues. Accordingly, the estimate of 24 components in A-gliadin may be minimal —there may be many more components when differences in neutral amino acids are taken into account.

We cannot rule out the possibility that some of the complexity of the A-gliadin mixture results from post-translational modifications. We know that A-gliadin is not a glycoprotein— it contains no carbohydrate (BERNARDIN *et al.*, 1976), but it is possible that some enzymatic modification of amide side chains could contribute differences to originally identical A-gliadin molecules although I am unaware of any evidence for this or any other type of post-translational modification of A-gliadin. It seems necessary, in consideration of the amino acid sequence differences and compositional differences in components of A-gliadin that we have observed, to conclude that multiple genes code for A-gliadin components. I think that an estimate of at least five copies of the gene (or parts of the gene if A-gliadin genes are broken up into parts as seems true of most eukaryotic genes) would be highly conservative (and allow for the possibility of some post-translational modification) and there may be as many as 100 copies. These conclusions are based on evidence for distinguishable components; until research into the DNA structure of the regions of the wheat genome that include gliadin genes is carried out, we have no way of knowing how many identical copies of A-gliadin genes there might be that code for products indistinguishable by protein separation techniques.

B. — *Speculative Model for Origin of Gliadins*

a) *Origin of multiple genes coding for A-gliadin*

It is difficult to provide more than a guess as to when the multiplication occurred that produced a complex of A-gliadin genes or whether this took place over a short or long period of time. I speculate, however, that this multiplication was connected with speciation of the *Triticinae*, the sub-tribe of the grass family (Gramineae) that includes the wild diploid species combined in polyploid wheats. I have shown some tentative evidence for the contribution of A-gliadin components to common wheat by *T. urartu* and we have not seen a similar complex in other diploids of the *Triticinae* that we have examined (Mecham and Kasarda, 1980); this may indicate that formation of the complex occurred in conjunction with divergence of the diploids from some common ancestor. Fossil evidence that provides evidence of when this divergence took place is not available, but Shewry *et al.* (1980) speculated that barley and wheat may have diverged about 16×16^6 years ago if the rate of evolution of the N-terminal portion of certain proteins common to these two species was about equal to that observed for the fibrinopeptide system (1 p. 100 change in amino acids per 1.1 million years —Doolitle, 1979), which is one of the fastest-evolving polypeptide systems characterized so far. The divergence of the species of *Triticinae* probably occurred within a few million years after the divergence of *Hordeum* from some common evolutionary line.

b) *Origin of the ancestral A-gliadin gene*

I have speculated that the multiplication of a gene that gave rise to the A-gliadin complex of components took place less than 16 million years ago. Because of the similarity of the components of the A-gliadin complex, however, it seems likely that the multiplied gene had already undergone considerable evolutionary change that distinguished it from some other gliadin genes, such as those coding for the γ_2-, γ_3 -gliadin components, which differ from A-gliadin in N-terminal sequence (Bietz *et al.*, 1977).

A possible mechanism for formation of this ancestral gliadin gene was suggested to me by the appearance in an ω-gliadin from the wheat variety *Justin* of the following amino acid sequence:

-PRO-GLN-GLN-PRO-TYR-PRO-GLN-GLN-PRO-TYR-

(Autran *et al.*, 1980*b*). The predominance of glutamine and proline in gliadins, combined with our observation of the repeated five member sequence shown above, leads me to speculate that reiteration of a short DNA sequence similar, but not necessarily identical, to the ω-gliadin repeat, produced the basis for the ancestral A-gliadin gene. Introduction of transcription controls at appropriate points in the sequence completed the process. This mechanism is similar to that proposed for the origin of genes for many other proteins that include highly repetitive sequences (Ycas, 1972; Black and Dixon, 1967; Kobayashi and Fox, 1978). In addition, Thompson and Murray (1979) have concluded from a consideration of the considerable variations in genome size that occur, even within a

single genus, among higher plants and from a consideration of the differences in gene organization that occur among these plants, that DNA sequence amplifications, rearrangements, and deletions are frequent events during evolution.

To illustrate this hypothesis, I have calculated the composition of A-gliadin on the basis of the expected mutation of the ancestral gene by using a simple model based on the following assumptions: (1) the reiterated DNA sequence consisted of 5 codons (CAGCAGCAGCCATAC) corresponding to the sequence -GLN-GLN-GLN-PRO-TYR-; this sequence was chosen on the basis of trial calculations; (2) only changes in amino acids occurred that resulted from single base changes of the fundamental codons; these are illustrated in figure 13; (3) the extent of mutation that has occurred is equivalent to the change in the proportion of glutamine in going from the unmutated protein to A-gliadin (43 p. 100); proline and tyrosine change in the same proportion; (4) only those changes that produce neutral

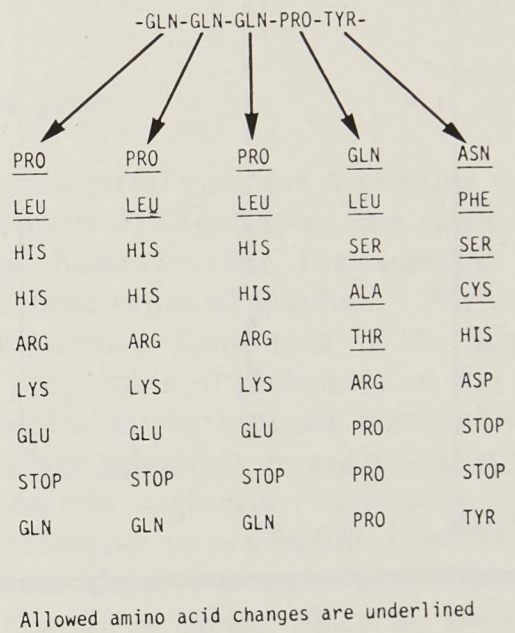

FIG. 13. — *Transformations resulting from single base changes in the codons of the fundamental repeating unit.*

amino acids became fixed; changes that produced charged amino acids disappeared; thus, the entire 43 p. 100 change in glutamine resulted only in the production of two amino acids, leucine and proline, in equal amounts, and (5) there were no functional constraints on mutation (except in the implied constraint of assumption 4). The results of the calculation are shown in Table 2 where they are compared with the actual composition of A-gliadin.

The agreement of the hypothetical A-gliadin composition with the actual composition is fair —perhaps even good— considering the partial validity af the assumptions made. The model correctly predicts that the three most predominant amino acids in A-gliadin are glutamine, proline, and leucine and also predicts that serine is more common than most other amino acids. The levels of proline and leucine are too high, however, and there is more tyrosine than serine in contrast to the actual composition. These failures seem to result from the limitations of some of the assumptions. In particular, the assumption (4) that only neutral amino acids became fixed does not reflect the actual situation. This assumption was necessary because otherwise the model would result in considera-

TABLE 2

The amino acid composition of A-gliadin compared with the hypothetical composition calculated for the ancestral gliadin polypeptide (-Gln-Gln-Gln-Pro-Tyr-)$_{n=55}$ *after 43 p. 100 of all amino acids have been transformed to other amino acids*

Amino acid	Amino acid compositions (residues/mole) A-gliadin	hypothetical
Gln	100 (*)	99
Pro	40	67
Leu	22	41
Tyr	9	31
Ser	15	11
Phe	10	6
Asn	8	6
Cys	5	6
Thr	4	5
Ala	7	5
Val	12	0
Ile	12	0
Gly	7	0
His	6	0
Arg	5	0
Met	3	0
Lys	1	0
Trp	1	0

(*) 108 (Gln + Glu) — 8 Glu.

bly more charged residues than are known to occur; gliadins are poor in charged side chains. This may reflect a functional constraint related to the necessity of packing gliadins quite closely in endosperm deposits as they are synthesized, with a consequent requirement for a low charge and a relatively non-polar nature. If glutamine were allowed to mutate, in a limited way, to those charged amino acids whose codons can result from single base changes in the glutamine codon (I chose only one codon for each amino acid although there are two for glutamine and tyrosine and four for proline), the levels of proline and leucine would be decreased. Allowance of two base changes (which is a common occurrence in the evolution of many proteins —see DOOLITTLE (1979) for review) would produce all other amino acids in A-gliadin. For example, single base changes can transform leucine codons (which would result from single base changes in the fundamental codons) to those of the following amino acids: isoleucine, valine, serine, phenylalanine, glutamine, proline, methionine, tryptophan, arginine, and histidine.

With regard to the overly-high proportion of tyrosine, the assumption that a fundamental unit of DNA coding for only the five specified amino acids was multiplied extensively in a short time may not be a good one. If, for example, the fundamental five codon unit doubled, one of the tyrosine codons was mutated to a phenylalanine codon, and then extensive reiteration of this ten codon unit took

place, an improved proportion of tyrosine and phenylalanine would result. This is merely one example of many adjustments to the simple model that might be made, but we (AUTRAN *et al.*, 1980*b*) have noted that transpositions in short amino acid sequences occur in comparisons of ω-gliadin N-terminal sequences; this indicates that DNA transpositions may play a significant role in the evolution of gliadin genes and limits the usefulness of further adjustments to my simple model based on point mutations.

If we again follow the assumption of SHEWRY *et al.* (1980) that changes in gliadin composition occur at about the same rate as that observed for the fibrinopeptide system, our assumption of 43 p. 100 change in the amino acid composition of the ancestral gene product since its origin (based on a best fit of the model) leads to an estimate that the reiteration occurred 47 million years ago. The outer limit for this event is probably defined by the appearance of flowering plants (Angiosperms) on the earth during the late Cretaceous period about 100 million years ago.

c) *Origin of other gliadin genes*

It is possible that the ancestral gene for A-gliadins was also the ancestral gene for all the gliadins, which have diverged from one another by way of duplications and mutations. Perhaps, however, the fundamental unit of DNA that was reiterated differed for different types of gliadins. For example, ω-gliadins do not differ much in their composition from that of the sequence -GLN-GLN-GLN-PRO-PHE. The higher proportion of glutamine in the composition of ω-gliadins in comparison with A-gliadins might indicate that the reiteration that produced their ancestral gene took place relatively recently —that they are relatively young proteins. Depending upon the ω-gliadin component considered (CHARBONNIER, 1974), the decrease in glutamine in ω-gliadins from that of the repeating unit suggested above ranges from about 8 to 25 p. 100 corresponding to a time of formation (if evolutionary change is taking place at about the rate found for the fibrinopeptide system) ranging from about 9 to 28 million years ago. This corresponds approximately to the time (16 million years) estimated by SHEWRY *et al.* (1980) since divergence of the lines of wheat and barley, based on changes in the largely homologous N-terminal amino acid sequences of an ω-gliadin from *T. monococcum* and a C-hordein from barley. This might also reflect the time of formation of ω-type (or B-type) prolamins although some time would have to be allowed for development of the common sequence, which is not that of a simple repeating sequence.

The alternative possibility is that ω-gliadins are as old as other gliadins, but that some functional constraint on their evolution (of which we are not aware) has prevented them from changing as rapidly as other gliadins. In connection with this, I refer to the earlier speculations of BOOTH and EWART (1969) who suggested that gliadins may have arisen from a single precursor by mutations, and that gliadins may have fewer structural constraints on their evolution in comparison with other proteins, such as enzymes. They also suggested that ω-gliadins may be primitive storage proteins because of their higher nitrogen content. Later, EWART (1972) suggested that, rather that being primitive types, ω-gliadins might actually be more highly evolved types of storage protein with selective advantage having resulted from trends toward more glutamine in the composition.

In my proposal, I suggest that different prolamins might have evolved from different precursors (more than one reiteration of a fundamental amino acid sequence) and that ω-gliadins are relatively young proteins that have not had time to undergo as much evolutionary change as other gliadins, such as α-gliadins. I emphasize that this is a highly speculative model. Only future research will enable us to sort out the most likely course of evolution that led to present day gliadins.

IV. — Conclusions

Wheat varieties may be divided into two types (Type 1 and Type 2) on the basis of major features of the gel electrophoretic patterns of their α-gliadins. Almost all characterization of α-gliadins has been of components derived from Type 1 wheat varieties.

A-gliadin and, probably, related α-gliadins from Type 1 wheat varieties aggregate specifically through secondary bonding forces to form fibrillar aggregates. This aggregation provides a useful model for studying the importance of conformationally-controlled fibril formation of other proteins in the dough system.

The A-gliadin fraction from Type 1 wheat varieties is complex and composed of many components, which result in part from coding by multiple gene copies. The genes coding for A-gliadin components may have been contributed to polyploid bread wheats (Type 1) by *T. urartu*. The multiplication that produced A-gliadin genes may have taken place in conjunction with speciation of *T. urartu* and its divergence from other members of the sub-tribe *Triticinae*.

A speculative model for formation of A-gliadin genes is based on the reiteration of relatively short DNA sequences (fundamental sequences) corresponding in length to only a few amino acid codons. Different fundamental sequences may have provided the basis for formation of different classes of gliadins. The reiteration of the fundamental sequence that gave rise to ω-gliadins may have taken place more recently than the reiteration that gave rise to α-gliadins.

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Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

Résumé

Structure et propriété de l'α-gliadine

En se fondant sur les traits principaux du diagramme électrophorétique de leurs α-gliadines, on peut séparer les variétés de blé en deux groupes (type I et type II). Presque tous les travaux ont porté sur la caractérisation des constituants des α-gliadines des variétés de type I.

L'A-gliadine et les α-gliadines (qui lui sont probablement apparentées) des variétés de type I s'assemblent spécifiquement par l'effet d'interactions secondaires pour former des agrégats fibrillaires. Cette agrégation semble dépendre de la conformation des protéines; elle fournit un modèle pour étudier le rôle de ce type d'agrégation fibrillaire dans le cas d'autres protéines intervenant dans la pâte.

La fraction A-gliadine des variétés de blé du type I comprend de nombreux constituants; cette complexité est due en partie au codage par des copies multiples d'un gène .Les gènes responsables ont peut-être été apportés aux blés polyploides panifiables par *Triticum vrartu*. La multiplication qui a produit les gènes de l'A-gliadine pourrait avoir été liée à la différenciation de *T. vrartu* en tant qu'espèce et à sa divergence des autres membres de la sous-tribu des *Triticinae*.

Un modèle hypothétique de la formation des gènes de l'A-gliadine est proposé, fondé sur la réitération de séquences d'ADN relativement courtes (séquences fondamentales), dont la longueur correspond à des codons de quelques acides aminés seulement. Des séquences fondamentales différentes pourraient être à l'origine de la formation de classes de gliadines différentes. La réitération de la séquence fondamentale qui est à l'origine des ω-gliadines pourrait être apparu plus récemment que la réitération à l'origine des α-, β- et γ-gliadines.

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Some physicochemical properties of Triticum vulgare β, γ and ω gliadins

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Summary

β, γ and ω-gliadins are 3 groups of constituents, visible in electrophoresis of total gliadin of *Triticum vulgare* at acid pH. They have been named by order of decreasing electrophoretic mobility.

In Cappelle wheat, and probably in other *Triticum vulgare*, since the total gliadins of these wheats give similar electrophoretic diagrams at acid pH, each of the β, γ and ω-groups makes up about 30, 30 and 15 p. 100 of the whole gliadin, respectively.

On the basis of the amino acid compositions of β, γ and ω-gliadins isolated from different wheats, no clear boundary can be drawn between the β and γ groups, whereas the ω-gliadins are distributed in 3 well defined groups.

The highest average hydrophobicities (1 200 Kcal/1 000 residues) were found for the slowest ω-group but the lowest average hydrophobicities (990 Kcal/1 000 residues) were also found for one of the 2 other ω-groups. β and γ-gliadins had intermediate average hydrophobicities (1 050-1 100 Kcal/1 000 residues).

The results concerning gliadin molecular weights are depending on the methods used for their determination. The molecular weight of a given gliadin determined by SDS-PAGE is sometimes 20 p. 100 higher than the molecular weight determined by gel filtration or analytical ultracentrifugation. If polyacrylamide gradient gel electrophoresis, gel filtration and analytical ultracentrifugation are considered as the most reliable methods, all β and γ-gliadins which have been isolated have molecular weights between 30 and 35 000. ω-gliadin molecular weights are twice higher.

Most of β and γ-gliadins have N-terminal amino acid sequences of the " α-type " (V-R-V-P-V-P-Q...) or " γ-type " (N/P)-(I/M)-(G/Q)-(V/Q)-(V/D)... The few known N-terminal sequences of ω-gliadins cannot be connected with any of these sequences.

The organ culture tests of toxicity carried out with peptic tryptic digests of β, γ and ω-gliadins have shown that β-gliadins are more noxious than γ-gliadins for coeliac patients. ω-gliadins, have been found unnoxious.

Key words : *Triticum Vulgare, endosperm, storage proteins, gliadins, physicochemical properties, coeliac disease.*

Introduction

Since an exhaustive review concerning wheat proteins has been published by KASARDA *et al.* (1976), some other gliadins have been isolated and characterized. Most of these gliadins were β and γ-gliadins (PATEY and WALDRON, 1976, BIETZ *et al.*, 1977, EWART, 1977*a*, 1977*b*, 1977*c*, SEXSON *et al.*, 1978, TERCÉ *et al.*, 1980).

Moreover, much more data are now available concerning the N-terminal amino acid sequences and the toxicity for coeliac patients of gliadins. These characteristics have been determined as well for gliadins isolated before 1976 as for gliadins isolated after this date. The other characteristics of *Triticum vulgare* β, γ and ω-gliadins recorded in this paper are: amino acid compositions, on the basis of which the average hydrophobicities have been computed, and molecular weights.

1. — *The α, β, γ and ω-gliadin groups of wheat*

Figure 1 shows the electrophoretic diagrams at acid pH of total gliadins of some wheats used as starting material for the isolation of β, γ and ω-gliadins described in the literature. The relative positions of β, γ and ω-gliadin groups are

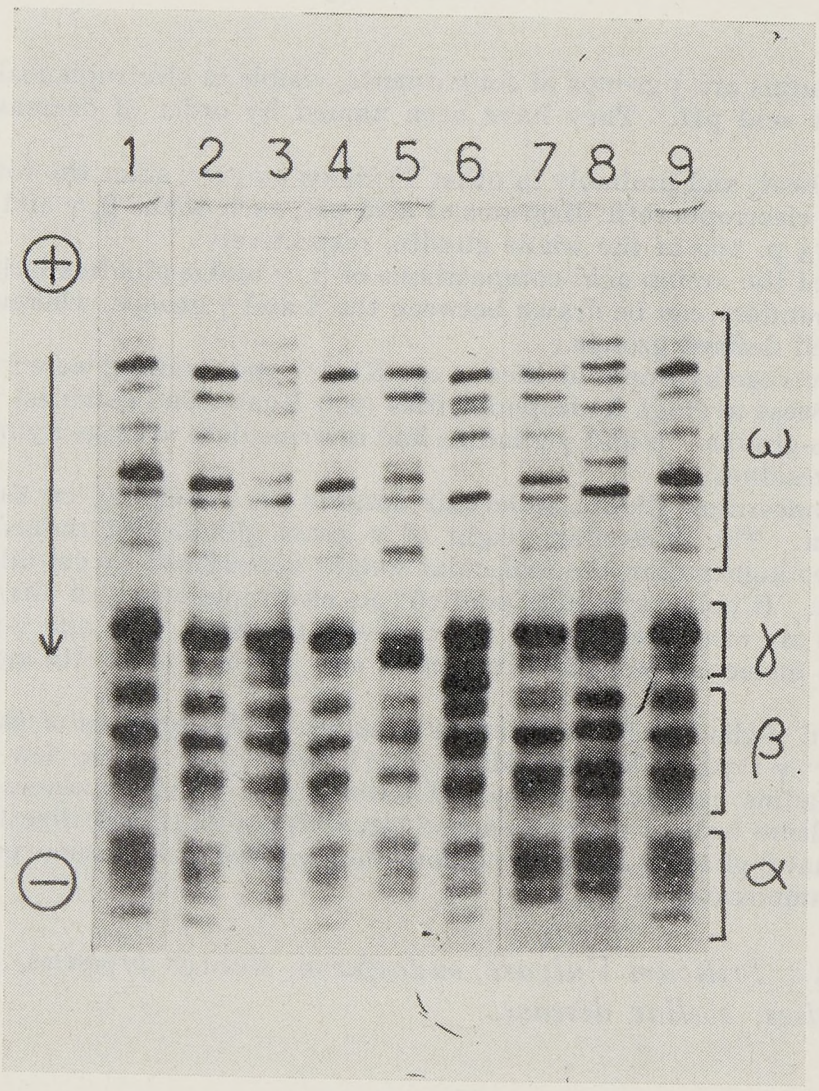

FIG. 1. — *Starch gel electrophoresis at pH 3.2 of total gliadins.*
1,4 and 9 = Cappelle, 2 Maris Widgeon, 3 = Ponca, 5 = Wichita, 6 = Marquis, 7 = Capitole and 8 = Kolibri.

shown on the right side. Inside a given group or subgroup, the electrophoretic constituents are numbered according to the order of their decreasing mobility. This nomenclature system has been proposed by WOYCHIK (1961). A clear boundary can be seen between ω and γ-gliadins but not between γ and β-gliadins. This is specially clear in the diagram of Marquis wheat which has been proposed by BUSHUK and ZILLMAN (1978) as reference wheat of a gliadin nomenclature system only based upon electrophoretic mobilities. In this paper, the gliadins will keep the names they received in the laboratories in which they have been isolated.

First, the amino acid compositions of β, γ and ω-gliadin groups will be compared on the basis of results concerning the gliadin fractions of Cappelle wheat separated by chromatography on SP-Sephadex C 50 (CHARBONNIER and MOSSÉ, 1980).

2. — *Amino acid compositions of gliadin fractions isolated ou SP-Sephadex C 50*

The electrophoretic diagrams of these gliadin fractions are presented on figure 2. Fractions 1 and 2 are only composed of ω and γ-gliadins, respectively. Fractions 3, 4 and 5 are composed of β-gliadins and fractions 7 and 8 of α-gliadins. Franction 6 only, is a mixture of fast-moving β-gliadins and slow moving α-gliadins.

The amino acid compositions of these 8 fractions are given in Table 1. The

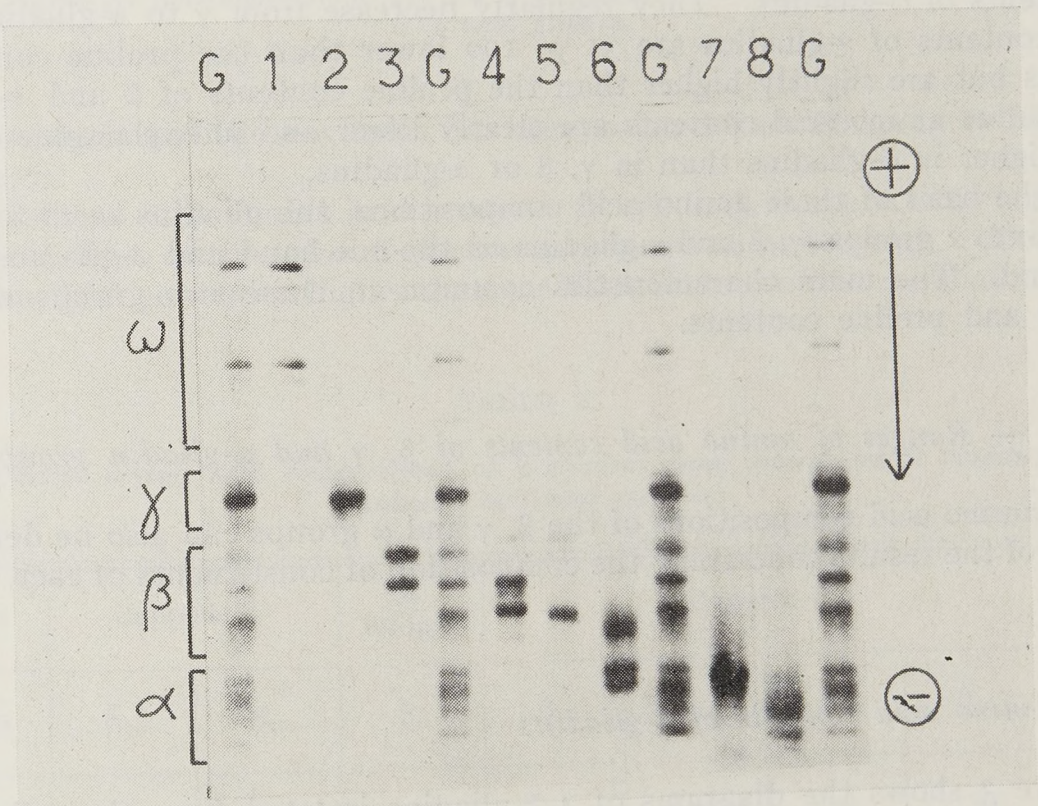

FIG. 2. — *Starch gel electrophoresis at pH 3.2 of gliadin fractions separated by ion exchange chromatography of total Cappelle gliadin (G) on SP-Sephadex C 50.*
1,8 = fraction numbers in the order of elution from the SP-Sephadex column.

comparison of these compositions is interesting because all these fractions have been isolated from the same wheat and on the other hand because these compositions have been determined in the same laboratory. As we shall see, a given gliadin may have several amino acid compositions, if these compositions have been determined in different laboratories.

TABLE I

Partial amino acid compositions of Cappelle wheat gliadin fractions isolated on SP-Sephadex (Residues/1 000 residues) (1)

	1	2	3	4	5	6	7	8
BAA (2)	20	29	32	36	38	40	44	50
Glx	501	403	405	394	395	383	373	370
Pro	229	175	161	164	155	153	160	151
1/2 Cys	4	24	25	25	33	27	23	26
Met	1	11	10	9	6	17	10	8
Phe	91	51	39	42	43	43	40	39

(1) CHARBONNIER et MOSSÉ (1980).
(2) Total of basic amino acids.

In Table 1, totals of basic amino acid (BAA) contents are regularly increasing from ω to α-gliadins. The Glx contents of γ-gliadins are 24 p. 100 lower than Glx contents of ω-gliadins. They regularly decrease from γ to α-gliadins. The proline contents of γ-gliadins are 31 p. 100 lower than the proline contents of ω-gliadins but are slightly higher than the proline contents of β and α-gliadins. Lastly, sulfur amino acid contents are clearly lower and phenylalanine contents clearly higher in ω-gliadins than in γ, β or α-gliadins.

On the basis of these amino acid compositions, the gliadins seem to be distributed into 2 groups: γ, β and α-gliadins on the one hand and ω-gliadins on the other hand. The main characteristics common to these two groups are their high Glx and proline contents.

3. — *Ranges of amino acid contents of β, γ and ω-gliadin groups*

The amino acid compositions of the β, γ and ω groups can also be defined on the basis of the results concerning the composition of constituents of each of these 3 groups.

3.1. — *Amino acid contents of β-gliadins*

Figure 3 shows the diagrams of 4 β-gliadins isolated from Cappelle wheat (TERCÉ *et al.*, 1980). Three of these β-gliadins are the major constituents of the three β-gliadin subgroups visible in starch gel electrophoresis. β_{13} is the major constituent of subgroup β_1, β_{22} is the major constituent of subgroup β_2 and β_{32} is the major constituent of subgroup β_3. β_{12} which migrates in front of β_{13} is a minor constituent of subgroup β_1. This gliadin has also been isolated by EWART (1977*b*).

The amino acid compositions of these 4 gliadins are given in Table 2. β_{11} has also been isolated from Cappelle by EWART (1977*c*). This gliadin migrates in front of β_{12}. The β_1-gliadin has been isolated from Maris Widgeon by PATEY and

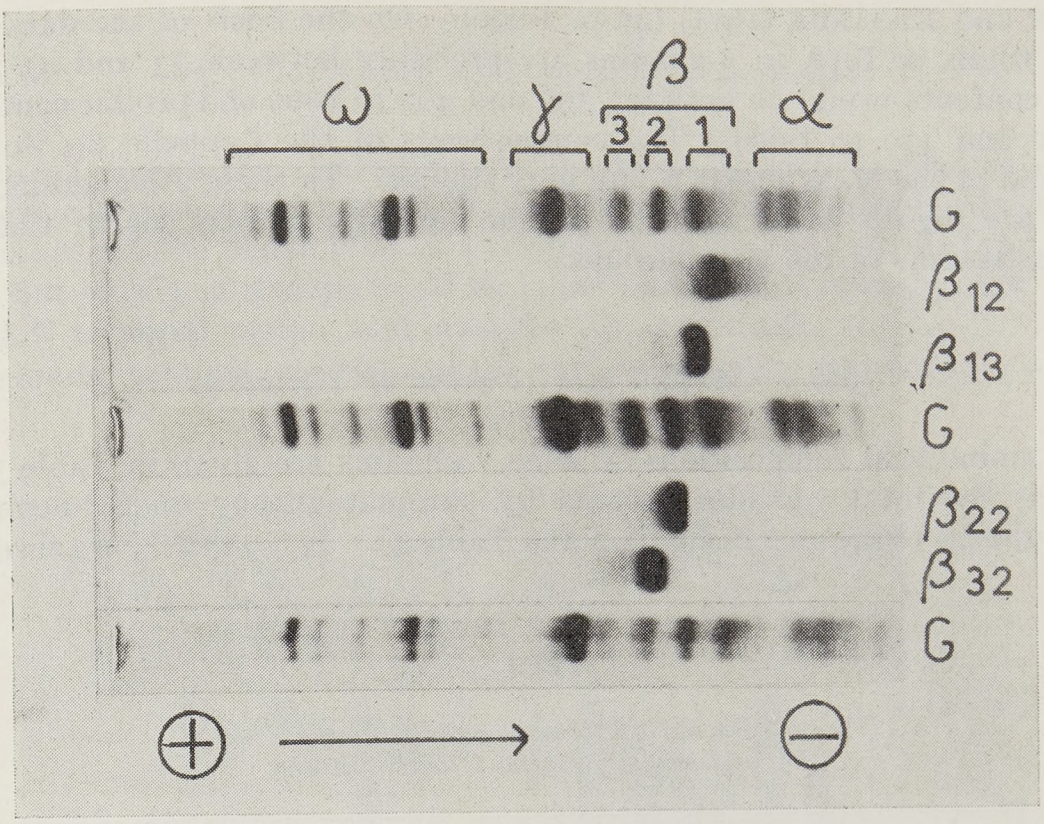

FIG. 3. — *Starch gel electrophoresis at pH 3.2 of β-gliadin fractions isolated from Cappelle wheat. G = total gliadin.*

WALDRON (1976) and the β_5-gliadin has been isolated from Ponca in Peoria (BIETZ *et al.*, 1977).

The total of basic amino acid contents (BAA) in β_5 is appreciably higher than the totals of BAA of the other β-gliadins and seems to us too high. As a matter of fact, this BAA content is higher than the BAA content of the α_{1b}-gliadin isolated

TABLE 2

Partial amino acid compositions of β-gliadins from several wheat varieties (residues / 1 000 total residues)

Wheats	Cappelle[1]		M. Widg[2]	Cappelle [3]				Ponca [4]
Gliadins	β_{11}	β_{12}	β_1	β_{12}	β_{13}	β_{22}	β_{32}	β_5
BAA . . .	43	39	42	41	38	34	33	48
Glx	354	349	339	365	410	389	400	396
Pro	166	186	190	161	150	169	158	158
1/2 Cys . .	23	23	25	26	19	23	23	18
Met	8	7	12	14	8	6	9	11
Phe	40	42	43	51	36	35	40	37

(1) β_{11} : EWART (1977*c*), β_{12} : EWART (1977*b*).
(2) PATEY and WALDRON (1976).
(3) TERCÉ *et al.* (1980).
(4) BIETZ *et al.* (1977).

by PLATT and KASARDA (1971) (45 p. 1 000). On the basis of the data given in Table 2, totals of BAA in β-gliadins are probably between 33 and 43 p. 1 000. The Glx contents would be between 350 and 400 p. 1000 and proline contents between 150 and 170 p. 1 000. The compositions of the Cappelle β_{12} isolated by EWART and of the M. Widgeon β_1 are very similar. In these 2 β-gliadins, the Glx contents are slightly lower and the proline contents slightly higher than in the other constituents of the β_1 subgroups.

3.2. — *Amino acid contents of γ-gliadins*

The amino acid compositions of some γ-gliadins are given in Table 3. Cappelle γ_T is the total γ-gliadin isolated by chromatography on SP-Sephadex of Cappelle gliadin (fraction 2 of figure 2 and Table 1). γ_1, γ_2 and γ_3 are three γ-gliadins isolated from Ponca wheat by HUEBNER *et al.* (1967). γ_{31} and γ_{32} have been isolated from Cappelle wheat by EWART (1977*a*). These 2 gliadins are constituents of the strong band visible in the γ-region of the diagram of Cappelle gliadin (fig. 1). Although they are two of the main constituents of the total γ-gliadin of Cappelle, 15 p. 100 differences are found between totals of BAA on the one hand and Glx contents on the other hand.

TABLE 3

Partial amino-acid compositions of γ-gliadins (Residues / 1 000 residues)

Wheats	Capelle (1)	Ponca (2)			Cappelle (3)	
Gliadins	γ_T	γ_3	γ_2	γ_1	γ_{31}	γ_{32}
BAA	29	32	33	29	34	35
Glx	403	391	375	399	351	338
Pro	175	187	178	153	196	183
1/2 Cys	24	19	21	21	26	26
Met	11	11	16	8	13	17
Phe	51	56	49	37	57	50

(1) CHARBONNIER and MOSSÉ (1980).
(2) HUEBNER *et al.* (1967).
(3) EWART (1977*a*). γ_{31} = 2nd peak, γ_{32} = 1st peak.

As the γ-gliadins isolated as well from Ponca as from Cappelle are slow moving γ, it is not possible to define with precision the ranges of amino acid contents of the whole γ-gliadin group.

For the slow moving γ-gliadins, the ranges of amino acid contents are probably those found for the γ_1, γ_2 and γ_3 gliadins isolated from Ponca. Totals of BAA would be between 29 and 33 p. 1 000, Glx contents between 375 and 400 p. 1 000 and proline contents between 155 and 190 p. 1 000.

3.3. — *Amino acid contents of ω-gliadins*

Figure 4 shows the electrophoretic diagrams of some ω-gliadins of known amino acid compositions which have been isolated from Cappelle wheat. These ω-gliadins seem to be distributed in 4 subgroups on the basis of the total ω-gliadin diagram but only 3 subgroups could be found on the basis of amino acid composition and N-terminal amino acid analysis (CHARBONNIER, 1974).

The amino acid compositions of the 3 major constituents of these 3 subgroups

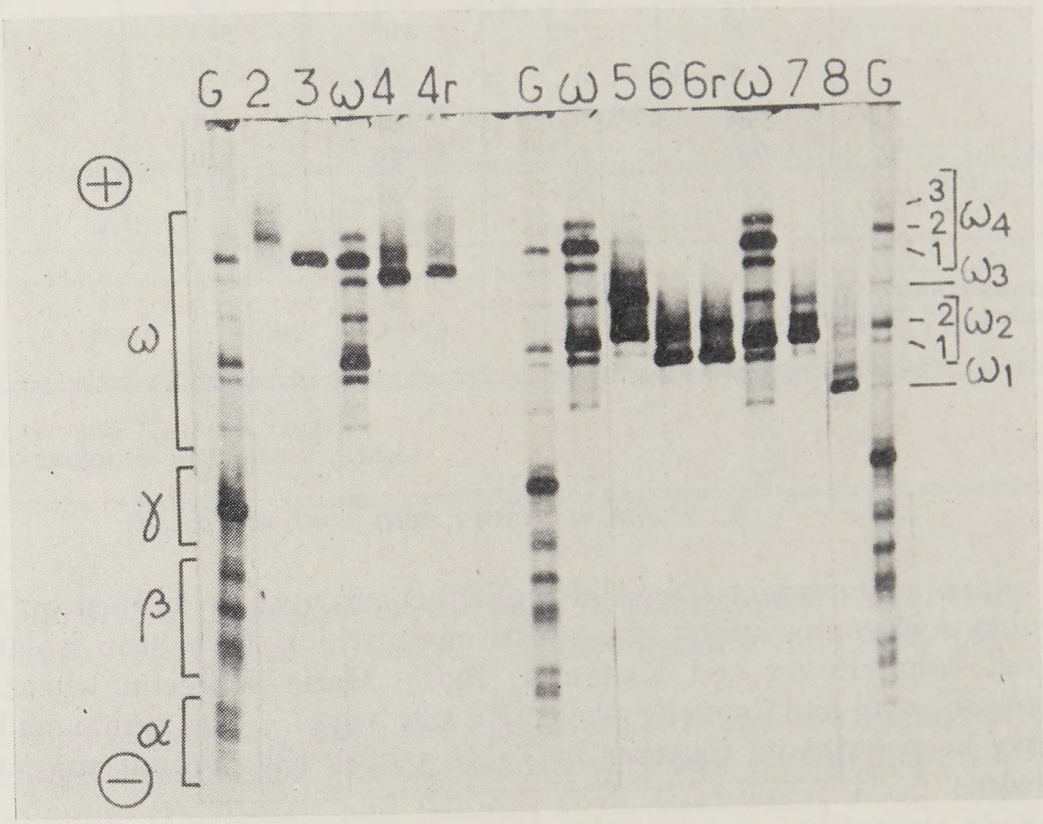

FIG. 4. — *Starch gel electrophoresis at pH 3.2 of ω-gliadin fractions separated by ion exchange chromatography of total ω-gliadin (ω) on SE Cellulose.*
G = total gliadin ; 2,8 = fraction numbers in the order of elution from the XSE-cellulose column.

(ω_1, ω_{2+3} and ω_4) are given in Table 4. ω_{42}-gliadin has also been isolated from Cappelle and Wichita by BOOTH and EWART (1969) who named it SP 22.

The differences of amino acid compositions between the 3 subgroups of ω-gliadins are clearly shown by the BAA contents, Glx and proline contents. Although ω_{42} and SP_{22} isolated from Cappelle are the same constituents, ± 10 p. 100 differences can be calculated between Glx and proline contents.

ω-gliadin compositions are characterized by high Glx and proline contents. The sum of the contents of these 2 residues makes up 75 p. 100 of the total number of residues of ω_2 and ω_4 (SP_2) subgroups. In these 2 subgroups, the sum Glx + Pro + Phe makes up more than 80 p. 100 of the total number of residues. As ω_2 and ω_4 subgroup constituents are free of sulfur amino acids, they have been named " athins " by BOOTH and EWART (1969).

TABLE 4

Partial amino acid compositions of ω-gliadins (Residues /1 000 residues)

Wheats	Cappelle (1)				Wichita (2)
Gliadins	ω_1	ω_{22}	ω_{42}	SP_{22}	SP_{22}
BAA	31	25	11	12	12
Glx	493	559	451	383	420
Pro	198	199	298	363	310
1/2 Cys	9	0	0	0	0
Met	3	0	0	0	0
Phe	85	96	88	78	84
Glx + Pro	691	758	749	746	730
Glx + Pro + Phe	776	854	837	824	814

(1) CHARBONNIER (1974).
(2) BOOTH and EWART (1969).

3.4. — α, β, γ *and* ω *amino acid content ranges*

The amino acid content ranges of α-gliadins can also be defined on the basis of the amino acid compositions of α-constituents which have been isolated from Golden Seal flour (PLATT and KASARDA, 1971), Maris Widgeon wheat (PATEY and WALDRON, 1976) and Cappelle wheat (EWART, 1976). The amino acid content ranges have been gathered together in Table 5 with the content ranges of β, γ and ω-gliadins.

The definition of the amino acid content ranges of ω-gliadins is based upon the compositions found for ω-constituents isolated from Cappelle wheat (CHARBONNIER, 1974).

TABLE 5

Ranges of gliadin amino acid contents (Residues /1 000 residues)

Gliadins	α	β	γ	ω	ω_1	ω_4
BAA	59-41	43-33	33-29	31	24-25	9-14
Glx	330-390	350-400	375-400	493	557-559	451-468
Pro	145-160	150-170	155-190	198	197-199	275-298
1/2 Cys	20-26	23-26	20-25	9	0	0
Met	7-10	6-14	8-16	3	0	0
Phe	36-40	35-50	37-56	85	96-103	88-94

Broad overlappings can be seen between α, β and γ amino acid contents whereas there are clear boundaries between ω_1 and γ groups on the one hand and beetwen each of the three ω-subgroups on the other hand.

4. — α, β, γ *and* ω-*gliadin average hydrophobicities*

The average hydrophobicities of α, β, γ and ω-gliadins were computed on the basis of their amino acid compositions, according to TANFORD (1962) and BIGELOW (1967). Results are presented in Table 6. Average hydrophobicities of α and γ-gliadins were computed on the basis of the compositions of total α (PLATT and KASARDA, 1971) and γ-gliadins (CHARBONNIER and MOSSÉ, 1980) respectively.

TABLE 6

Gliadin average hydrophobicities
(Kcal/1 000 residues)

Gliadins	α_T(1)	β_{13}	β_{22}	β_{32}	γ_T(2)	ω_1	ω_{22}	ω_{42}
$H\phi_{ave}$ . . .	1 976	1 007	1 095	1 056	1 069	1 052	991	1 204

(1) PLATT and KASARDA (1971).
(2) CHARBONNIER and MOSSÉ (1980).

Although ω_{42} and ω_{22} have the highest and the slowest average hydrophobicities, respectively, these 2 gliadins are the very first eluted gliadins from hydrophobic columns (POPINEAU and GODON, 1978, CALDWELL, 1979) what enhances the discrepancy between apparent hydrophobic properties of gliadins and their average hydrophobicities.

5. — *Molecular weights of* β, γ *and* ω-*gliadins*

5.1. — β-*gliadiu molecnlar weights*

The most often used methods of gliadin molecular weight determination are analytical ultracentrifugation, use of amino acid compositions, polyacrylamide gel electrophoresis in the presence of SDS, polyacrylamide gel electrophoresis and gel filtration. Results concerning β-gliadin molecular weights are given in Table 7. The molecular weight of a given gliadin is obviously depending on the method used for the determination. For instance, the molecular weights of β_{12}, β_{13} and β_{32} determined by SDS-PAGE are 20 p. 100 higher than the molecular weights determined by gel filtration. The discrepancy between gel filtration molecular weights of M. Widgeon and Cappelle β_1 is probably due to the unsuitableness of acetic acid as solvent for molecular weight determination of gliadins by gel filtration.

TABLE 7

Molecular weights of β-gliadins
($M \times 10^{-3}$)

Wheats	Cappelle		Cappelle[3]			M. Widg.[4]	Ponca	
Gliadins	β_{11}	β_{12}	β_{13}	β_{22}	β_{32}	β_1	β_5	β_6
UC								33[6]
AA	42[1]	38 [2]				28		
SDS-PAGE	42[1]	37 [2]	41,3	36,5	38,5			35-40[5]
G-PAGE		30,6[3]	34,3	33,8	34,9			
GF		39,2[3]	34,3	32,5	32	40		

UC = ultracentrifugation, AA = amino acid analysis.
SDS-PAGE = polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, G-PAGE = polyacrylamide gradient gel electrophoresis, GF = gel filtration.
[1] EWART (1977*c*). [2] EWART (1977*b*) [3]. TERCÉ *et al.* (1980). [4] PATEY and WALDRON (1976). [5] BIETZ *et al.* (1977). [6] SEXSON *et al.* (1978).

5.2. — *γ-gliadin molecular weights*

The molecular weights of Ponca γ_1 and γ_3-gliadins (Table 8) determined by SDS-PAGE are also 20 p. 100 higher than molecular weights determined by ultracentrifuge. This discrepancy between SDS-PAGE and ultracentrifugation results has already been pointed out by HAMAUZU *et al.* (1974, 1975). As ultracentrifugal molecular weights are independent of the shape of the gliadin molecules, they are considered as closest to the true molecular weights (SEXSON *et al.*, 1978). However, HAMAUZU and YONEZAWA (1978) suggested that ultracentrifugal mole-

TABLE 8

Molecular weights of γ-gliadins
(kilodalton)

Wheats Gliadins	Ponca			Cappelle[6]	
	γ_1	γ_2	γ_3	γ_{31}	γ_{32}
UC	30.3[1]	34.6[2]	34.7[1]		
AA	32[3]	36.5[4]	42.6[4]	44	38
SDS-PAGE	36.5[5]		44[5]	42	38.6

[1] SEXSON and WU (1972). [2] SEXSON *et al.* (1978). [3] HUEBNER *et al.* (1967). [4] BIETZ *et al.* (1977). [5] BIETZ and WALL (1972) [6] EWART (1977*a*). γ_{31} = 2nd peak, γ_{32} = 1st peak.

cular weights of gliadins might be underestimated because the partial specific volume computation on the basis of amino acid compositions might be unsuitable for proteins with so high glutamine and proline contents.

With the exception of molecular weights determined by SDS-PAGE and of molecular weights based upon amino acid compositions which are adjusted to SDS-PAGE results (EWART, 1977*a*, 1977*b* and 1977*c*), all molecular weights of β and γ-gliadins are between 30 and 35 000.

5.3. — *ω-gliadin molecular weights*

The molecular weights of ω-gliadins determined as well by ultracentrifugation as by SDS-PAGE or gel filtration are approximately twice higher (Table 9) than β and γ molecular weights (Tables 7 and 8). However, an ultracentrifugal

TABLE 9

Molecular weights of ω-gliadins
(kilodalton)

Wheats Gliadins	Cappelle(1)		Brevor(2)		Cappelle(1)	Wichita(3)
	ω_1	ω_{22}	ω_x	ω_y	ω_{42}	SP_{22}
UC						78,7
AA						74,7
SDS-PAGE . . .			78,1	69,3		
GF	73,1	80,5			65,3	

(1) CHARBONNIER (1974). (2) BIETZ and WALL (1972). (3) BOOTH and EWART (1969).

molecular weight of 27 000 was claimed for a mixture of ω-gliadins (HAMAUZU *et al.*, 1974). These authors suggested that the lack of disulfide bonds in ω-gliadins might allow these gliadins to take a more expanded conformation than other gliadins. However, all molecular weight determinations of gliadins by gel filtration or SDS-PAGE are carried out with reduced proteins which are therefore disulfide bond free.

6. — *Gliadin N-terminal amino acid sequences*

6.1. — *β and γ N-terminal sequences*

β_5 and γ_1-gliadins isolated from Ponca have N-terminal sequences identical with the N-terminal sequence of the α_2-gliadin isolated from golden seal flour (KASARDA *et al.*, 1974). This N-terminal sequence has also been found in Cap-

pelle β_{22} (HUET, unpublished results). Moreover, the N-*t* residue of all β-gliadins isolated from Cappelle, with the exception of β_{33} is a valine (TERCÉ *et al.*, 1980). Therefore, all these gliadins have probably a N-terminal sequence similar to the α_2 N-*t* sequence the N-*t* residue of which is a valine. As this N-terminal sequence has also been found in all α-gliadins isolated from Ponca wheat (BIETZ *et al.*, 1977) it was named " α type " N-*t* sequence by AUTRAN *et al.* (1979). This α type sequence is not only common to most of the α and β constituents and some of the γ-gliadins of a given wheat variety but is also common to the α and β constituents of different varieties (see Table 10). On the contrary, in addition to the

TABLE 10

N-terminal sequences of α, β, γ and ω-gliadins

Wheats	Gliadins	Residues
		1 2 3 4 5 6 7 8 9 10
Golden seal flour(1) . . .	α_2	
Scout 66(1)	α_T	
Ponca(2)	α_8 to α_{12}	Val-Arg-Val-Pro-Val-Pro-Glu-Leu-Glu-Pro
Ponca(2)	β_5	
Cappelle(3)	β_{22}	
Ponca(2)	γ_1	
		1 2 3 4 5 6 7 8 9 10
Ponca(2)	γ_2	Asn-Ile-Gly-Val-Asp-Pro-Trp-Gly-Gln-Val Pro Gln Val Gln Leu
Ponca(2)	γ_3	Asn-Met-Gly-Val-Asp-Pro-Trp-Gly-Gln-Val Pro Gln Gln Val Gln Gln
Kolibri(4)	γ	Val-Ile-Val-Gln-Val-Arg-Gln-Leu-Gln-Val
		1 2 3 4 5 6 7 8 9 10
Justin(5)	ω_{1D}	Lys-Glu-Leu-Gln-Ser-Pro-Gln-Gln-Ser-Phe
Justin(5)	ω_{1B}	Ser-Arg-Leu-Leu-Ser-Pro-Arg-Gly-Lys-Glu

(1) KASARDA *et al.* (1974). (2) BIETZ *et al.* (1977). (3) HUET (unpublished results). (4) PATEY *et al.* (1975). (5) AUTRAN *et al.* (1980, unpublished results).

α type N-terminal sequence found in Ponca γ_1, two other N-terminal sequences have been found in γ_2 and γ_3 of the same wheat variety. Moreover, the N-terminal end of a Kolibri γ-gliadin is different from the 3 sequences found in Ponca γ-gliadins (PATEY *et al.*, 1975).

6.2. — ω *N-terminal seqnences*

The only two N-terminal amino acids of the α, β and γ N-terminal ends are valine and asparagine. As the only three N-*t* amino acids found in Cappelle ω-gliadins were Alanine, Serine and Threonine (CHARBONNIER, 1974), these glia-

dins have probably N-terminal sequences different from those found for α, β and γ-gliadins. In fact the 2 N-terminal sequences of JUSTIN wheat ω-gliadins determined by AUTRAN *et al.* (1980) show that the only homology between the N-terminal sequence of these ω-gliadins and the α, β and γ N-*t* sequences is a Proline residue in position 6 (see Table 10).

7. — *Toxicity of β, γ and ω-gliadins for cœliac patients*

Total gliadin is known to promote impairments of the intestinal mucosa correlated with malabsorption of almost all nutritients, in coeliac patients. (DICKE, 1950, VAN DE KAMER *et al.*, 1953, VAN DE KAMER and WEIJERS, 1955). The noxious antigenic structure had been shown to be present as well in α as in β-gliadins, on the basis of immunological study of gluten proteins (BECKWITH and HEINER, 1966). However, in spite of these results, α-gliadins have been thought for some times to be the only noxious gliadins (KENDALL *et al.*, 1972, EVANS and PATEY, 1974).

In 1977, JOS *et al.* have shown on the basis of organ culture test results, that β-gliadins are as noxious as α-gliadins and that even γ-gliadins are slightly noxious. ω-gliadins only would be unnoxious (Table 11).

TABLE 11

In vitro *toxicity of PT digests of gliadins*(1)

	PT digests of					
	o	Gliadin	α	β	γ	ω
Coeliac patients (33 flat biopsies) . . .						
Reparation	9				2	2
No reparation	1	1	3	1	1	3
Impairment		4	5	3	2	
Extensive necrosis		7	7	3	1	
Controls (5 normal biopsies)						
No change		3	3			
Slight change						

(1) JOS *et al.* (1977), PT = peptic tryptic.

As the length of peptic-tryptic peptides has been shown to be a direct function of their Proline content (CHARBONNIER *et al.*, 1980), the differences of toxicity between gliadins can be considered as partially correlated with the differences between their proline content since only peptides with molecular weights between 5 000 and 10 000 have been shown to be noxious (DISSANAYAKE, 1973 and 1974, JOS and REY, 1975).

Conclusion

The obvious conclusion of this short review concerning the physico-chemical properties of β, γ and ω-gliadins is that on the basis of their amino acid compositions, molecular weights, N-terminal amino acid sequences and toxicity for coeliac patients — properties correlated with the structure of gliadins — these proteins are distributed into 2 groups: one group is composed of α, β and γ-gliadins and the other group is composed of the three ω-gliadin subgroups.

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Résumé

Quelques propriétés physicochimiques des β, γ et ω gliadines de Triticum vulgare

Les β, γ et ω-gliadines sont 3 groupes de constituants mis en évidence par électrophorèse de gliadine totale de *Triticum Vulgare* à pH acide. Ils sont nommés par ordre de mobilité électrophorétique décroissante.

Dans le Blé Cappelle, ainsi probablement que dans d'autres *Triticum Vulgare*, puisque toutes les gliadines totales de ces Blés donnent des diagrammes électrophorétiques semblables à pH acide, chacun des groupes β, γ et ω représentent respectivement environ 30, 30 et 15 p. 100 de la gliadine totale.

D'après les compositions en acides aminés de β, γ et ω-gliadines isolées à partir de différents Blés, il n'y a pas de frontière bien nette entre les groupes β et γ, alors que les ω-gliadines se répartissent en 3 groupes bien définis.

C'est le groupe d'ω-gliadines le plus lent qui a les hydrophobicités moyennes les plus élevées (1 200 Kcal/1 000 résidus), l'un des 2 autres groupes d'ω ayant les hydrophobicités moyennes les plus faibles (900 Kcal/1 000 résidus). Les hydrophobicités moyennes des ω et γ-gliadines ont des valeurs intermédiaires (1 050-1 100 Kcal/1 000 résidus).

Les résultats concernant les poids moléculaires des gliadines dépendent des méthodes utilisées pour leur détermination. Le poids moléculaire d'une gliadine donnée déterminé par SDS-PAGE est quelquefois de 20 p. 100 supérieur à celui déterminé par filtration sur gel ou ultracentrifugation analytique.

Si on considère l'électrophorèse en gradient de gel de polyacrylamide, la filtration sur gel et l'ultracentrifugation analytique comme les méthodes les plus fiables, les poids moléculaires de toutes les β et γ-gliadines isolées se situent entre 30 et 35 000. Les poids moléculaires des ω-gliadines sont 2 fois plus élevés.

La plupart des β et γ-gliadines ont des séquences N-terminales du « type α » (V-R-V-P-V-P-Q...) ou du « type γ » (N/P) — (I/M) — (G/Q) — (V/Q) — (V/D...). Les quelques séquences N-terminales connues d'ω-gliadines n'appartiennent à aucun de ces 2 types.

Les tests de toxicité par culture d'organes effectués avec des hydrolysats pepsiques-trypsiques de β, γ et ω-gliadines ont montré que les β-gliadines sont plus toxiques pour les sujets intolérants au gluten que les γ-gliadines. Seules les ω-gliadines semblent dépourvues de toxicité.

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Hydrophobic properties of the gliadins of some varieties of Triticum vulgare wheats

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Summary

Hydrophobic interaction chromatography has been applied to the study of surface hydrophobicity of gliadins on four *Triticum vulgare* varieties (three good quality wheats: " Magdalena ", " Manitoba ", and " Bezostaïa ", and one variety unsuitable for breadmaking: " Maris Huntsman ").

Binding capacity of gliadins on Phenyl- and Octyl-Sepharose CL_4B gels, their elution patterns and the electrophoretic (starch gel) composition of the fractions thus separated, have been compared.

The elution diagrams and the electrophoretic patterns of the fractions show the same general features; however varietal differences can be pointed out. Gliadins cover clearly a wide range of surface hydrophobicities; such a variability should be related to differences in functional properties.

It was not possible to find specific common characteristics for good quality wheats opposing them to the bad one. However, in presence of low concentrations of NaCl, the gliadins of one high quality wheat appear to be fairly more hydrophobic than those of the unbakable wheat. The impossibility to link gliadin composition, hydrophobicity and breadmaking quality could be due therefore to ill-chosen analytical conditions.

Key words : *wheat — gliadins — hydrophotricity.*

Introduction

Rheological properties of wheat gluten depend on the structure of its constitutive proteins and of the strength of the interactions between proteins and between proteins and lipids. Several observations suggest that the hydrophobic interactions are important. Shortly:

— the properties of solubility of the gluten components in organic solvents and detergent solutions (Wakar *et al.*, 1975, Kobrehel and Bushuk, 1977);

— the high content in apolar amino acids, related to the rheological properties of dough (LASZTITY 1975);

— the association of non polar lipids with gluten proteins (CHUNG and TSEN 1975);

— the visco-elastic properties of the gluten modified by addition of hydrocarbons (PONTE *et al.* 1967).

The presence of hydrophobic areas on the surface of the gliadins was first proved on α-gliadins by GREENE and KASARDA (1971) and confirmed by recently published results (POPINEAU and GODON 1978, CALDWELL 1979, CHUNG and POMERANZ 1979). In other respects varieties of Triticum vulgare wheats are differentiated by the electrophoretic analysis of the gliadins (AUTRAN and BOURDET 1975) and relations between gliadin components and technological value of wheats are investigated (ROUSSET and BRANLARD 1977).

Therefore we compared surface hydrophobicity of gliadins extracted from some varieties of *Triticum vulgare* wheats.

Material and methods

Four varieties of *Triticum vulgare* wheats were compared. They were: BEZOSTAIA, MAGDALENA, MANITOBA, MARIS HUNTSMAN.

Gliadins were extracted from *n*-pentane defatted gluten by a mixture of water and dioxane (60/40 v/v).

They were analyzed by hydrophobic interaction chromatography (HIC). This technique of chromatography was applied only recently to gluten proteins (POPINEAU and GODON 1978, CALDWELL 1979, CHUNG and POMERANZ 1979). It fractionates proteins according to the importance of the hydrophobic areas located at the surface of the molecules (HOFSTEE 1975) by means of different hydrophobic interactions between proteins and a gel matrix on which hydrophobic groups have been grafted. The strength of the interactions depends on the surface hydrophobicity of the proteins, on the hydrophobicity of the gel, and on the composition of the eluant. Cross-linked agaroses are often use das matrix. The elution is generally achieved by modifications of the solvent resulting in a direct weakening of the hydrophobic interaction between the protein and the gel (modification of the ionic strength, of the ions, of the pH) or in an increase of its affinity for the solvent (decreasing the polarity of the solvent). In some cases the elution process implies a change in the conformation of the protein, so that the importance or accessibility of the hydrophobic areas is decreased (biospecific desorption).

HIC techniques have been carefully reviewed in recent papers (SHALTIEL 1975, HOFSTEE 1976, YON 1978, OCHOA 1978).

The gliadins were separated using modifications of the pH and decreasing the polarity of the solvent to achieve the elution. Experimental conditions were as follows:

Phenyl-Sepharose CL 4B (Pharmacia). Column : length 180 mm, diameter 12.7 mm

Sample 150 mg gliadin dissolved into aluminum lactate buffer pH 3.6.

Elution: — aluminum lactate buffer pH 3.6 (60 ml);
— linear gradient of ethanol (0 to 60 p. 100) in 0.02 N Ammonia (480 ml);
— 0.02 N Ammonia, 60 p. 100 ethanol, 3 M urea.

Flow rate: 20 ml h^{-1}.

The composition of the fractions was determined by starch gel electrophoresis (12 p. 100 starch-aluminum lactate buffer pH 3.1 — 3 M urea).

Some complementary experiments have been performed on Octyl-Sepharose CL 4B (Pharmacia) as indicated below:

Column: 2 ml Octyl-Sepharose CL 4B.

Sample : 20 mg gliadin dissolved into 0.02 N acetic acid with or without 0.025 M NaCl.

Elution: — 0.02 N acetic acid with or without 0.025 M NaCl;
— 0.02 N ammonia;
— 0.02 N ammonia 20, 40, 60 p. 100 ethanol.

The amount of protein eluted by each solvent was determined. It gives a quantitative estimation of the hydrophobic binding of gliadins.

Results and discussion

I. — *General features of the fractionation of gliadins by HIC on Phenyl-Sepharose CL 4B*

Magdalena was taken as an example. Four main groups of components are present on the elution profile (fig. 1).

— One eluted by aluminum lactate buffer, but composed of non protein material. So all gliadins were bound to the gel in this buffer.
— A large amount of proteins (from 50 to 60 p. 100) was eluted by the raise of the pH. We separated it into three fractions.
— A series of peak and shoulders, that we divided into six fractions representing about 5, 7, 8, 8, 3 and 3 p. 100 respectively of the gliadins in the case of Magdalena. They were eluted by a progressive decrease of the solvent polarity. The main peaks (6 and 7) were eluted by 36 and 41 p. 100 ethanol.
— A last fraction was eluted in presence of 3 M urea.

The recovery was about 85 p. 100.

It appears thus that: the surface hydrophobicities of gliadins cover a wide range and therefore these proteins could have largely different functional properties. On the whole, their surface hydrophobicity is fairly high: their elution from the gel needed an important pH jump and high ethanol concentrations. Through the electrophoretic patterns of the fractions (fig. 2) we can try to compare the hydrophobicity of the gliadin components. The first constatation is that α and β-gliadin groups gather proteins with very different surface hydrophobicity: α and β components are found both in the less and the most hydrophobic fractions.

ω and γ-gliadins appear to be more homogenous with regard to surface hydrophobicity. However, a certain degree of separation was achieved among them by HIC. With the major part of α and some β-gliadins, ω-gliadins are the first proteins eluted; indeed the pH jump alone was sufficient to wash them out. This behaviour is a problem, as already prointed out by CALDWELL (1979), since other facts suggest on the contrary, that ω-gliadins are among the most hydrophobic gliadins. Some results obtained by other methods confirm that (POPINEAU and GODON, paper submitted to Cereal Chemistry). ω-gliadins were fractionated by HIC into two groups corresponding to the slow and fast electrophoretic components. Most of γ-gliadins were eluted with an ethanol concentration comprised

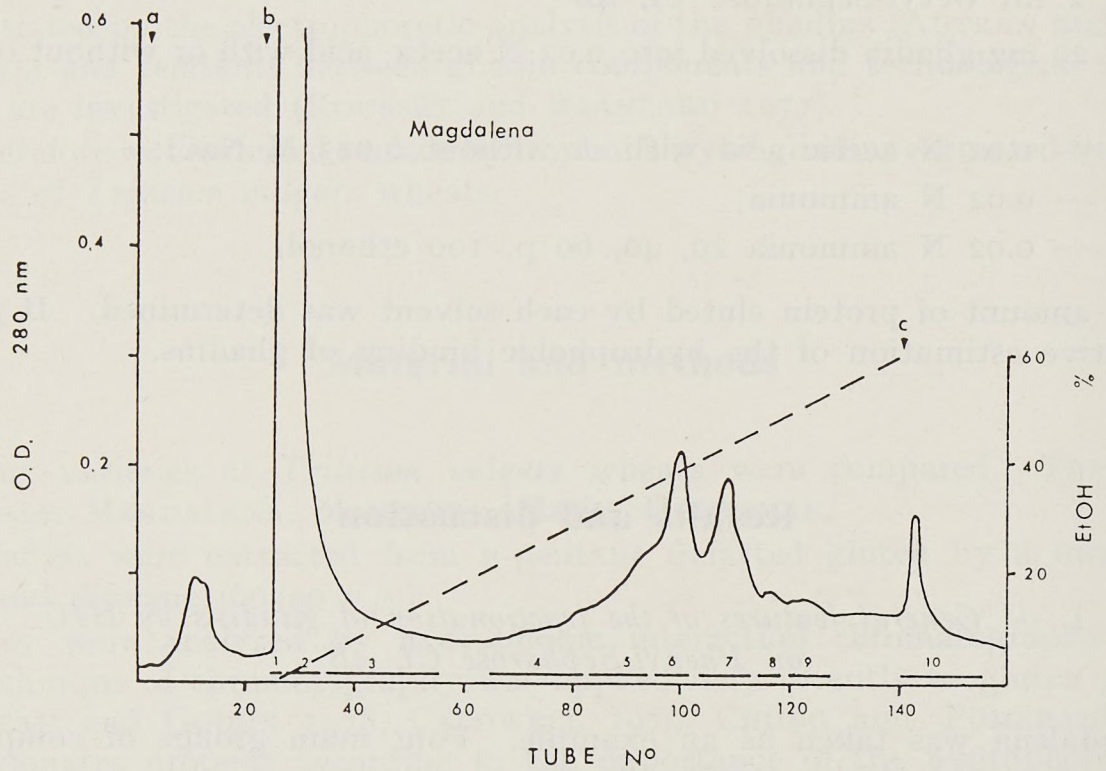

FIG. 1. — *Élution profile of gliadins of Magdalena separated by hydrophobic interaction chromatography on Phenyl-Sepharose CL 4B. Conditions of elution : a.) aluminium lactate buffer pH 3.6, b.) linear gradient of ethanol in 0.02 N ammonia (slope indicated by dashed line), c.) 0.02 N ammonia-60 p. 100 ethanol — 3 M urea.*

between 30 and 41 p. 100. So they present a rather high surface hydrophobicity. The γ 65 band in starch gel electrophoresis is found mainly in fractions 6 and 7; but, in fact, polyacrylamide gel electrophoresis according to BUSHUK and ZILLMAN (1978) resolved it into two components: the slower one appeared in peak 6, the faster one in peak 7.

II. — *Comparison of elution profiles of four Triticum vulgare wheat varieties*

The elution profiles show similar features (fig .1, 3, 4 and 5).

— All gliadins are absorbed on the gel in aluminium lactate buffer.
— An important amount (ca. 50 p. 100) is eluted by the pH jump.
— Several fractions are eluted between 25 and 45 p. 100 ethanol.
— Some gliadins need addition of urea for elution.

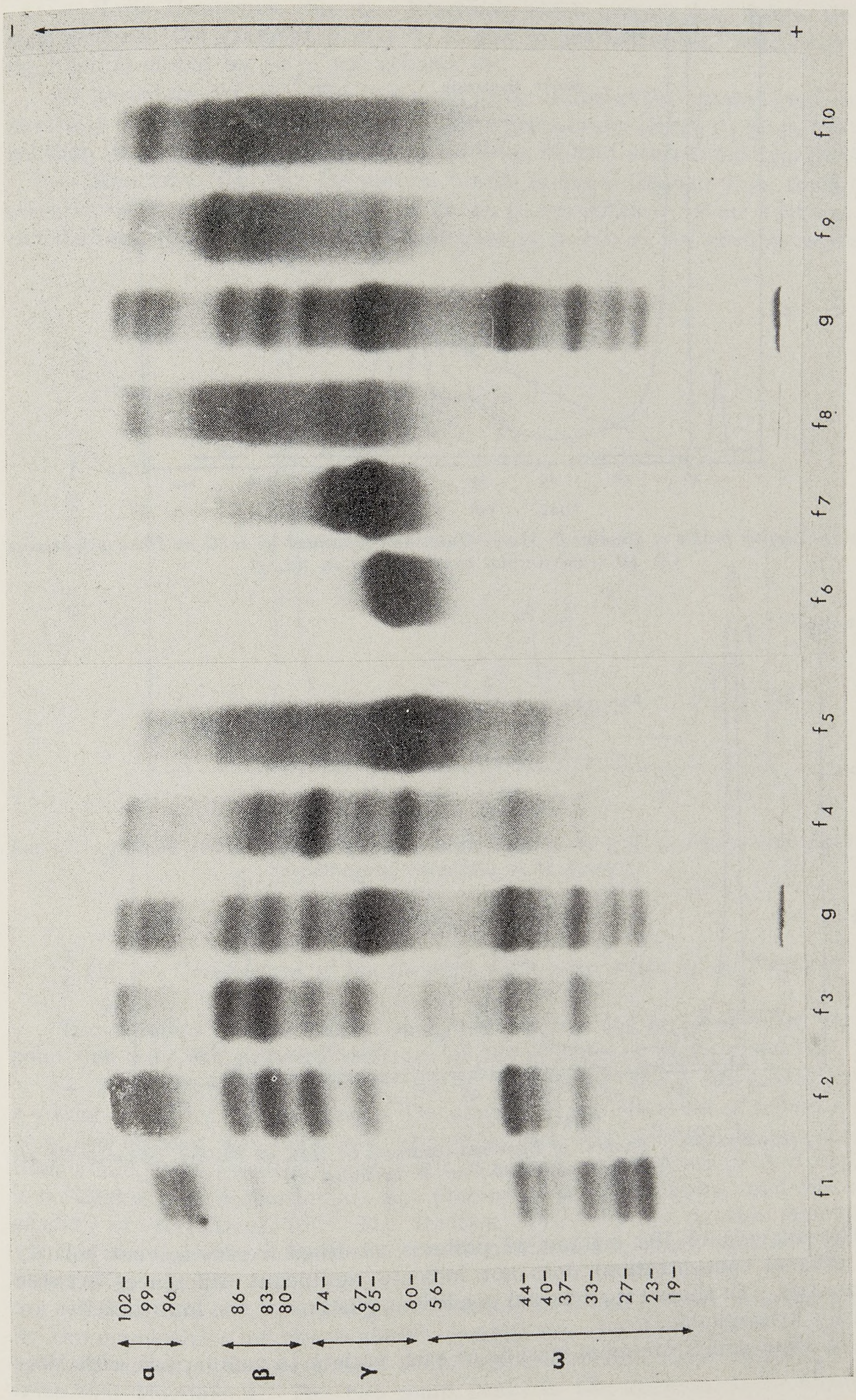

FIG. 2. — *Electrophoretic pattern of gliadin fractions of Magdalena separated by HIC on Phenyl-Sepharose CL 4 B (12 p. 100 starch — aluminium lactate buffer — 3 M urea — pH 3.1).*

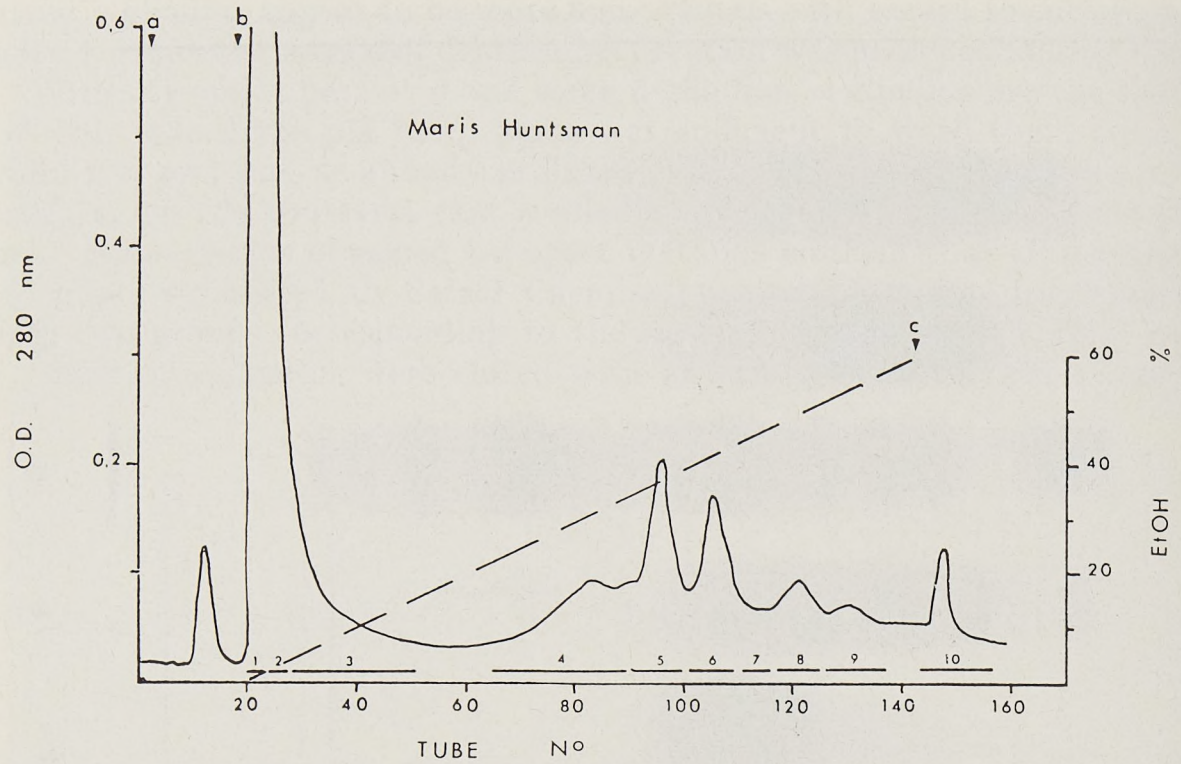

FIG. 3. — *Elution profile of gliadins of Maris Huntsman separated by HIC on Phenyl-Sepharose CL 4B (experimental conditions as in fig. 1).*

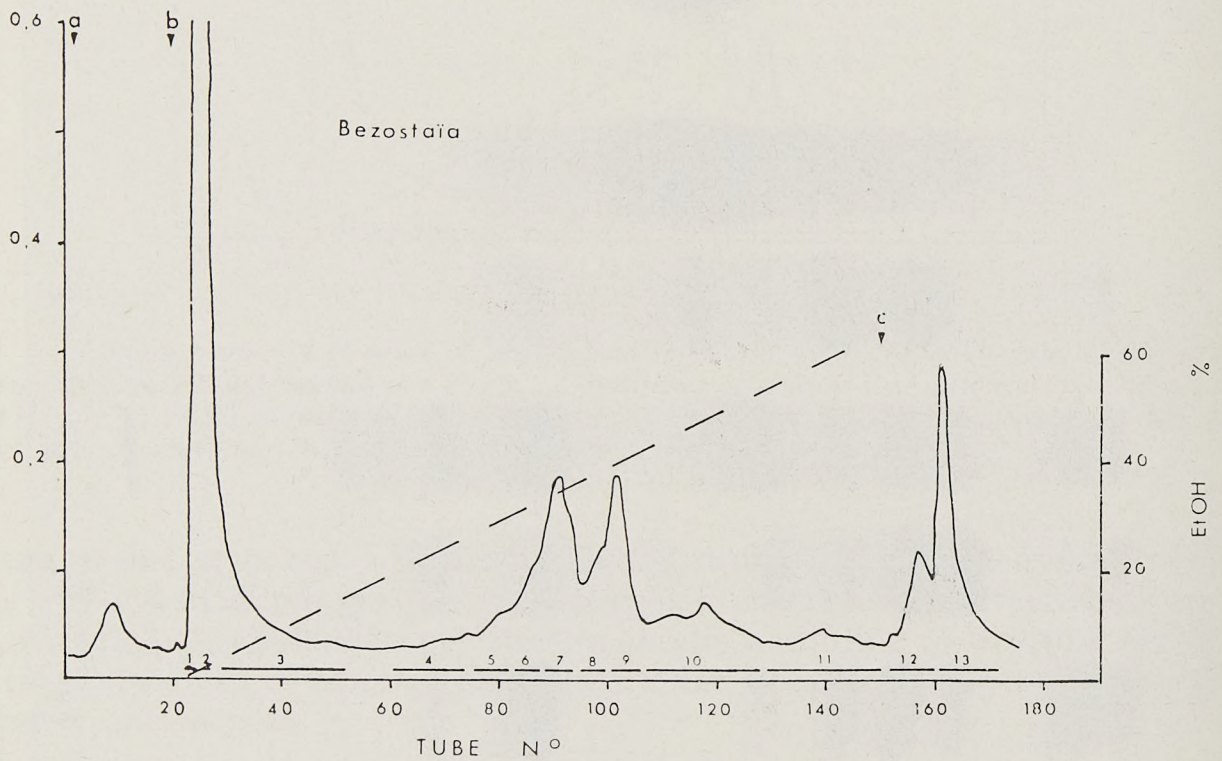

FIG. 4. — *Elution profile of gliadins of Bezostaïa separated by HIC on Phenyl-Sepharose CL 4B (experimental conditions as in figure 1).*

The analysis of the amount of proteins adsorbed versus solvent polarity (i. e. ethanol concentration) does not indicate significant differences between the varieties. In these experimental conditions gliadins of the four varieties are similarily hydrophobic.

Yet some points are more specific of each variety in comparison with Magdalena (fig. 1).

— Maris Huntsman (fig. 3). Fractions which precede and follow the two main peaks in the gradient (i. e. 4, 7, 8, and 9) are better separated, especially the fraction eluted by 50 p. 100 ethanol (8).

— Bezostaïa (fig. 4). The two main peaks eluted in the ethanol gradient can be clearly divided into four fractions. More proteins are eluted by 50 p. 100 ethanol than in the case of Magdalena and addition of urea washed out two fractions.

— Manitoba (fig. 5). Its elution profile is more different than that of the other varieties in the range from 25 to 45 p. 100 ethanol. Four fractions were eluted there; the most important counts for 11 p. 100 of the gliadins deposited.

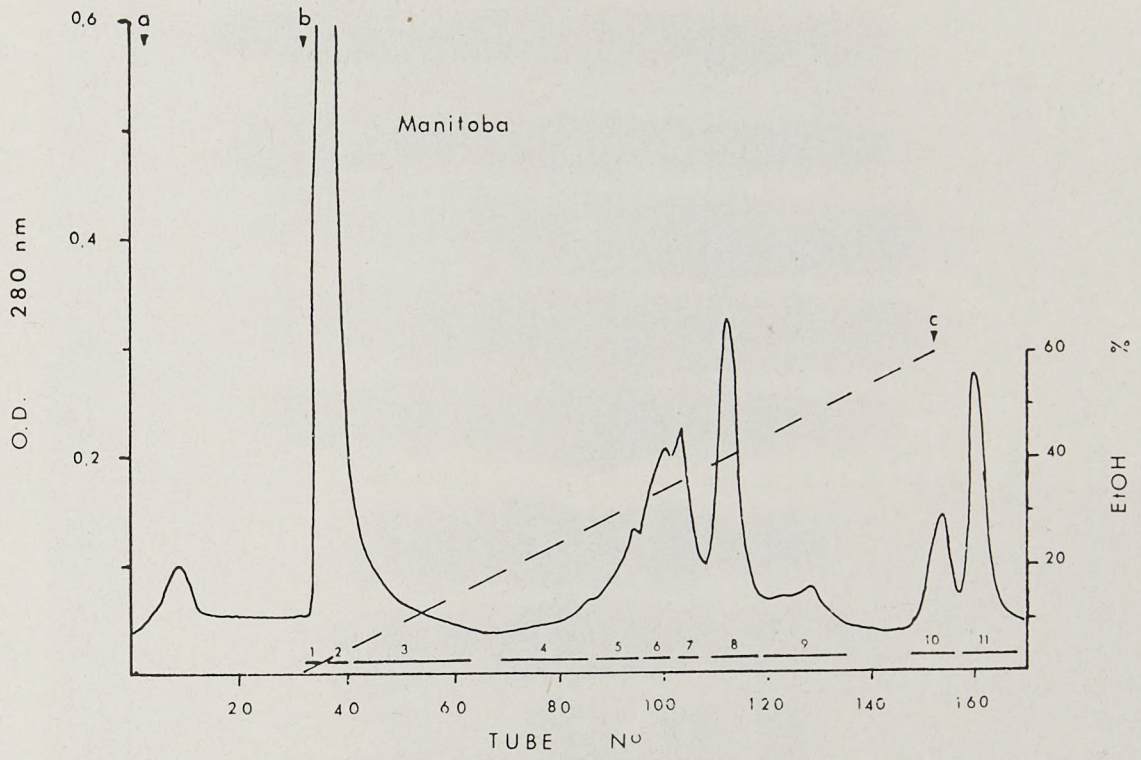

FIG. 5. — *Elution profile of gliadins of Manitoba separated by HIC on Phenyl-Sepharose CL 4B (experimental conditions as in figure 1).*

III. — *Comparison of the electrophoretic composition of the fractions*

The detailed composition of each fraction will not be described. We shall point out only the principal features of the gliadins of each variety.

— Maris Huntsman (fig. 6). As for Magdalena, the slower ω-gliadins and 2 α were eluted by the pH jump. The amount of γ-gliadins eluted between 0 and 10 p. 100 ethanol was very low (except for one fast γ-gliadin in 3). Thefractions 5 and 6 are essentially composed of γ-gliadins, but β are present in 5. It is noticeable that β-gliadins are not present in the homologous fractions of the gliadins of Magdalena (6). The fraction eluted by 50 p. 100 ethanol (8), which is more specific of the elution profile of Maris Huntsman, contains β-gliadins principally.

— Bezostaïa (fig. 7). The first two fractions (1 and 2) differ from one another by the composition of the ω-gliadin components mainly, and by α too. The poor separation of those fractions could explain this difference with Maris Huntsman and Magdalena. The two main peaks in the gradient are composed of

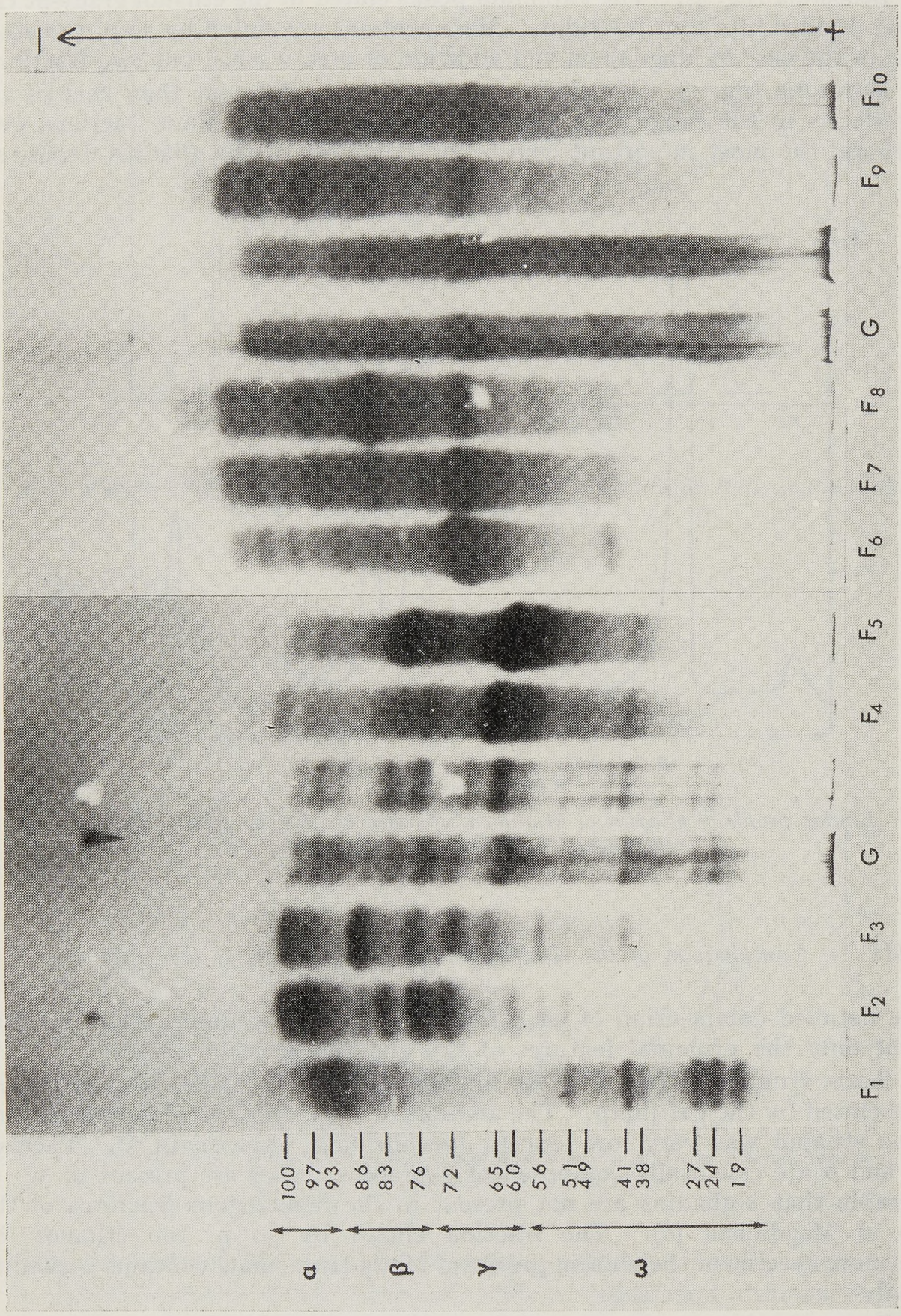

FIG. 6. — *Electrophoretic pattern of gliadin fractions of Maris Huntsman separated by HIC on Phenyl-Sepharose CL 4 B (experimental conditions as in figure 2).*

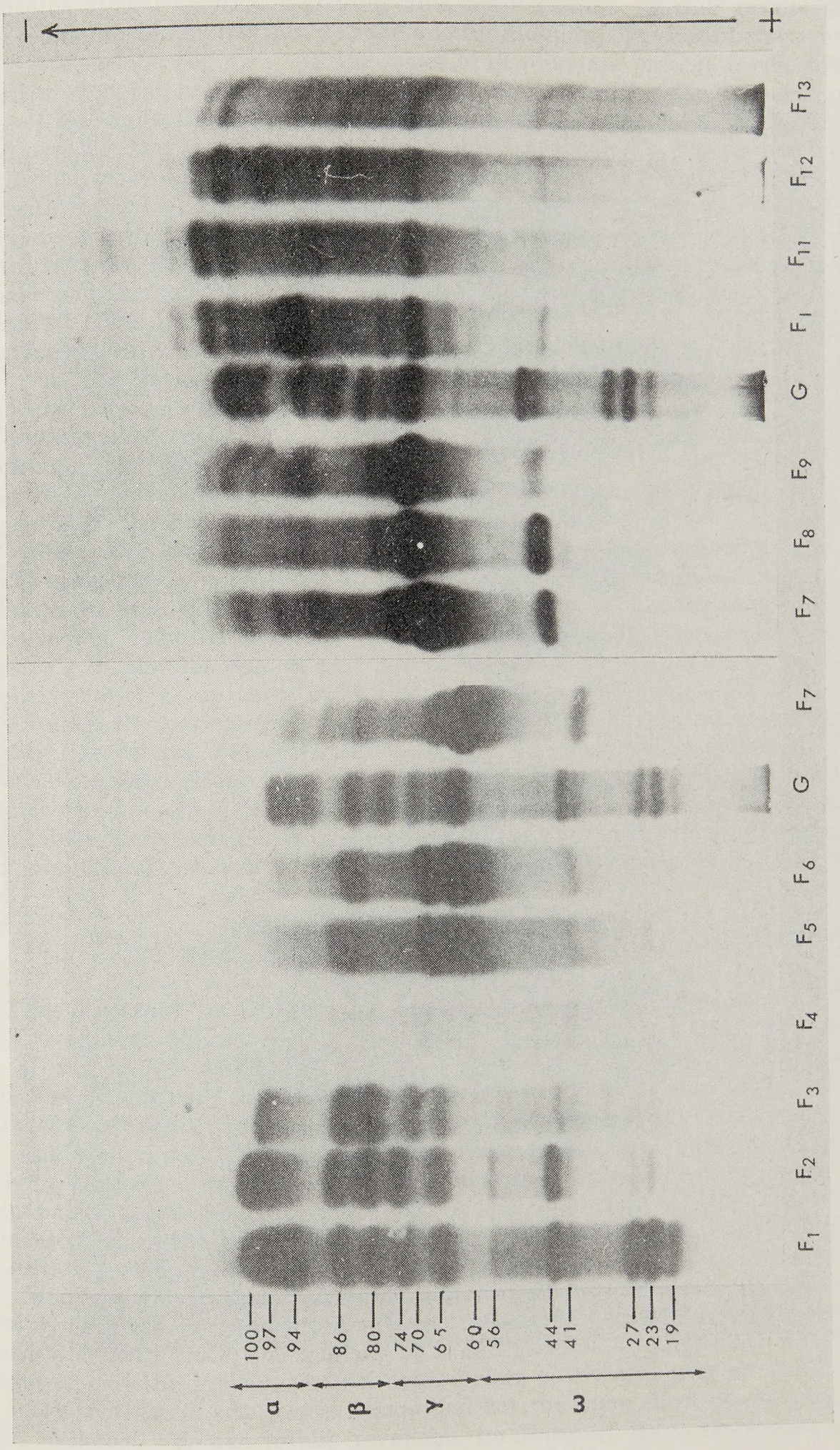

FIG. 7. — *Electrophoretic pattern of gliadin fractions of Bezostaïa separated by HIC on Phenyl-Sepharose CL 4B (experimental conditions as in figure 2).*

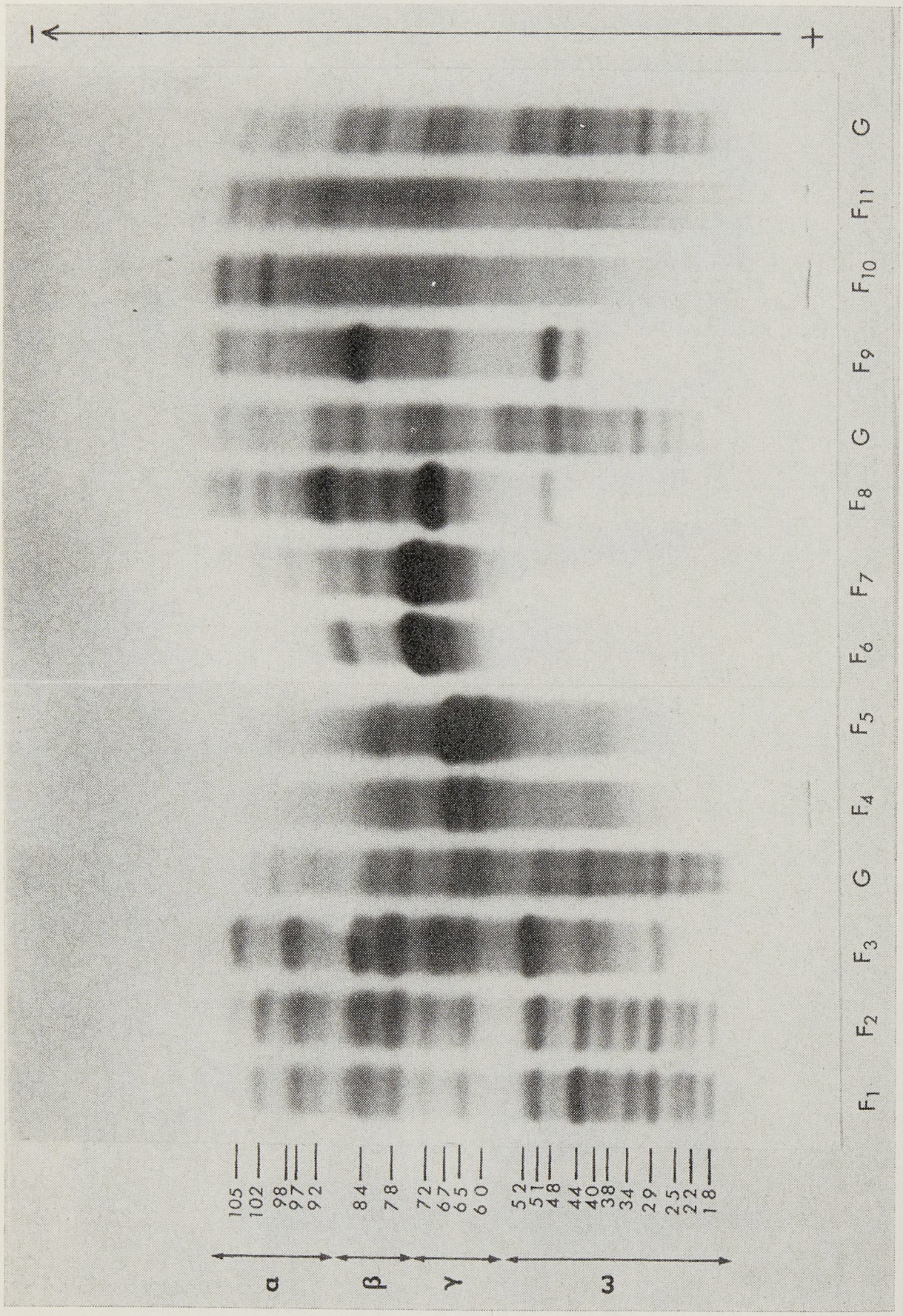

FIG. 8. — *Electrophoretic pattern of gliadin fractions of Manitoba separated by HIC on Phenyl-Sepharose CL 4B (experimental conditions as in figure 2).*

γ-gliadins appearing with β-gliadins and one ω-gliadin. PAGE results showed that γ-gliadins of the fraction 7 have a lower mobility than hat of fraction 9.

— Manitoba (fig. 8). All the types of gliadins are present in the first two fractions, but the amount of γ-gliadin is there very small. The presence of fast

— Manitoba (fig. 8). All the types of gliadins are present in the first two fractions, but the amount of γ-gliadin is there very small. The presence of fast ω-gliadins with one γ and α-gliadins that are absent in 1 and 2 (α 98 α 105) is characteristic of 3. The splitting of ω 51 and ω 52 into to two distinct fractions is noticeable. γ-gliadins are the principal components of the fractions eluted between 30 and 45 p. 100 ethanol. But the proteins present in 5, 6 and 7 have a higher mobility than those of 8. A β-gliadin is present too in those fractions. The fraction 9 is specific of this variety because of the presence of a strongly absorbed ω-gliadin (ω 44) with a β-gliadin (β 78).

Our HIC fractionation results show that surface hydrophobicities of gliadins are very various. The gliadin groups based on their electrophoretic mobility (Woychik *et al.*, 1961) are composed of proteins which have different hydrophobicity. Probably this electrophoretic classification should not be related directly to the functional properties of proteins, which depend essentially on the properties of the surface of the macromolecules.

Although components are present in several fractions, γ-gliadins appear to be the most homogenous group for they were all eluted in a narrow range of ethanol concentration, whatever was the variety. We found likewise only two groups of α-gliadins. The distribution of the β and of the ω-gliadins is more different from a variety to another one: in the case of Manitoba and Bezostaïa an ω-gliadin is strongly absorbed, on the contrary of the other ω-gliadins.

Among the varieties we compared, three wheats have a good breadmaking quality (Magdalena, Bezostaïa, Manitoba) and the fourth is an unbakable one by French standards (Maris Huntsman). The wheats of good quality have no clear specific common characteristics opposing them to the bad wheats, although significant differences exist between some fractions of each variety. But the comparison was difficult because of the complexity of the composition of the gliadins.

IV. — *Comparison of the binding of gliadins to Octyl-Sepharose CL 4B*

Big differences in hydrophobicity between gliadins of Magdalena and Maris Huntsman were pointed out by the measure of their binding to Octyl-Sepharose CL 4B in presence and absence of NaCl (fig. 9). When gliadins were dissolved and deposited in 0.02 N acetic acid, absorption of those of Magdalena and Maris Huntsman are quite identical, except for the amount of strongly absorbed gliadins.

Addition of 0.025 M NaCl promoted an increase of the binding of the gliadins of both varieties. But it was far more important for Magdalena than for Maris Huntsman. This difference in binding remained constant whatever was the solvent used subsequently to elute the gliadins. Thus it appears that the difference in surface hydrophobicity concerned all the gliadins.

Moreover the increased absorption of the gliadins of Maris Huntsman was entirely reversed by the elution with acetic acid without NaCl. If deposited in acetic acid with NaCl, the amount of bound gliadins of Magdalena was higher, whatever was the solvent, than if deposited in acetic acid without NaCl.

The increase of salt concentration had not the same effect on the gliadins of both varieties. It was totally reversible for those of Maris Huntsman but the

hydrophobic properties of the gliadins of Magdalena were irreversibly enhanced. This phenomenon could be due to an important modification of the conformation or of the aggregation state of the macromolecules. Thus, when salt is added to the solvent, surface hydrophobicity of the gliadins of the good quality wheat is higher than that of the gliadins of the bad one. Our results disagree with those of

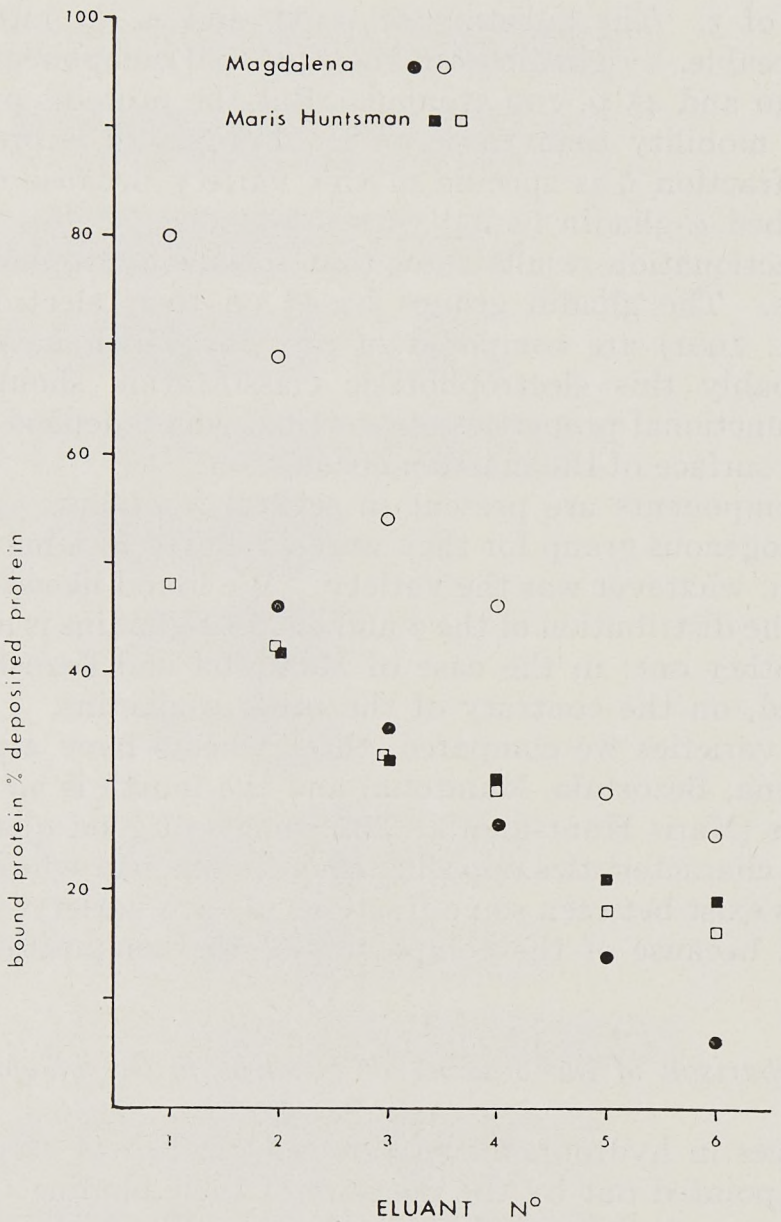

FIG. 9. — *Comparison of the binding of gliadins of Magdalena and Maris Huntsman to Octyl-Sepharose CL 4B.*

Eluant no. 1 0.02 N acetic acid 0.025 M NaCl,
no. 2 0.02 N acetic acid,
no. 3 0.02 N ammonia,
no. 4 id. 20 p. 100 ethanol,
no. 5 id. 40 p. 100 ethanol,
no. 6 id. 60 p. 100 ethanol.

CHUNG and POMERANZ (1979) who reported that gliadins are more hydrophobic for a bad than for a good wheat; but they are confirmed by preliminary results obtained on two varieties of *Triticum durum* wheats of good and bad quality (GODON and POPINEAU, paper to be published in *Agronomie*.

Conclusion

Surface hydrophobicities of the gliadins of four varieties of *Triticum vulgare* wheats were compared by hydrophobic interaction chromatography. We obtained a classification of the gliadins which differs from the groups defined according to their electrophoretic mobility; gliadins belonging to a same electrophoretic group have different surface hydrophobicity. A classification based on hydrophobic properties sould be related to the quality, since functional properties of proteins depend on their binding capacity. However, we could not so far find a precise relation between hydrophobicity of peculiar gliadin components and the technological value of wheats. This could be due to the experimental conditions not suited to this aim. Indeed, in other conditions (addition of salt) we could show that Magdalena gliadins (good breadmaking quality) are more hydrophobic than those of Maris Huntsman (unsuited for breadmaking). This study should be continued on a greater number of varieties and in more convenient experimental conditions.

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Résumé

Hydrophobicité des gliadines de quelques variétés de blé tendre

L'hydrophobicité de surface des gliadines de quatre variétés de blé tendre (Madgalena, Bezostaïa et Manitoba, blés de bonne qualité, Maris Huntsman, blé impanifiable) a été étudiée par chromatographie d'interaction hydrophobe sur Phenyl- et Octyl-Sepharose CL 4B. Les diagrammes d'élution des protéines, la composition des fractions (déterminée par électrophorèse en gel d'amidon) ainsi que la capacité de fixation sur les gels hydrophobes ont été comparés.

Si pour les quatre variétés de blé tendre, l'allure générale des diagrammes d'élution et des diagrammes électrophorétiques des fractions repérées présentent des similitudes, on a pu mettre en évidence quelques caractéristiques propres à chaque variété. On a confirmé la grande variabilité de l'hydrophobicité de surface des gliadines, qui doit se traduire par la diversité de leurs propriétés fonctionnelles. Il n'a pas été possible de dégager des caractéristiques spécifiques communes aux trois blé de bonne qualité et de trouver une différence très significative entre ceux-ci et le blé impanifiable. Cependant en présence de faibles concentrations en chlorure de sodium, les gliadines d'un blé améliorant sont nettement plus hydrophobes que celles d'un blé impanifiable. L'impossibilité de relier composition des gliadines, hydrophobicité et valeur technologique, peut donc n'être due qu'à des conditions d'analyse mal adaptées à la recherche de cette relation.

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SESSION III

Gliadines du blé :
Nomenclature et identification variétale des blés

Ann. Technol., agric., 1980, **29** (2), 207-212.

Wheat cultivar identification by high voltage gel electrophoresis

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Summary

A rapid electrophoretic method for wheat cultivar identification is described. Thin (0.75 mm) 6 p. 100 polyacrylamide gels with aluminium lactate-lactic acid buffer (pH 3.1) are used for the separation of wheat gliadins. Electrophoresis of 40 samples can be achieved in onlv 40 min at 1 000 V.

Key words : *electrophoresis — cultivar identification — gliadins.*

Introduction

With the potential of polyacrylamide gel electrophoresis (PAGE) of gliadin proteins for wheat cultivar identification having been reported (BUSHUK and ZILLMAN, 1978) (DUCROS and WRIGLEY, 1979), a suitable electrophoretic technique for the investigation of Canadian wheat cultivars in our laboratory was sought. In the selection of a procedure, points considered were: simplicity of apparatus assembly and gel formation, sample capacity, electrophoresis run time, resolving capabilities of the system and cost of gels. The method finally adopted is described in this paper.

Materiels and methods

Gliadin Extraction

A ground sample (Udy Cyclone Mill) or a pulverized kernel (hammer) was vortexed in a capped tube with three times its weight of 70 p. 100 aqueous ethanol and left to extract for 1 h at room temperature. The ground samples were then centrifuged for 10 min at 150,000 g at room temperature. The single kernel

samples were not centrifuged. An aliquot of the supernatant was removed and mixed with twice its volume of electrophoresis buffer containing 65 p. 100 sucrose.

The apparatus used for PAGE (Fig. 1) is the Bio-Rad Model 220 dual vertical slab cell (Mississauga, Ont., Canada). The gel slab is cast between parallel glass plates separated by polyvinyl chloride vertical side spacers of various thicknesses. Gel dimensions are 120 mm long by 140 mm wide and either 0.75 or 1.5 mm thick. A Teflon comb is used to form twenty wells per gel. Temperature is controlled

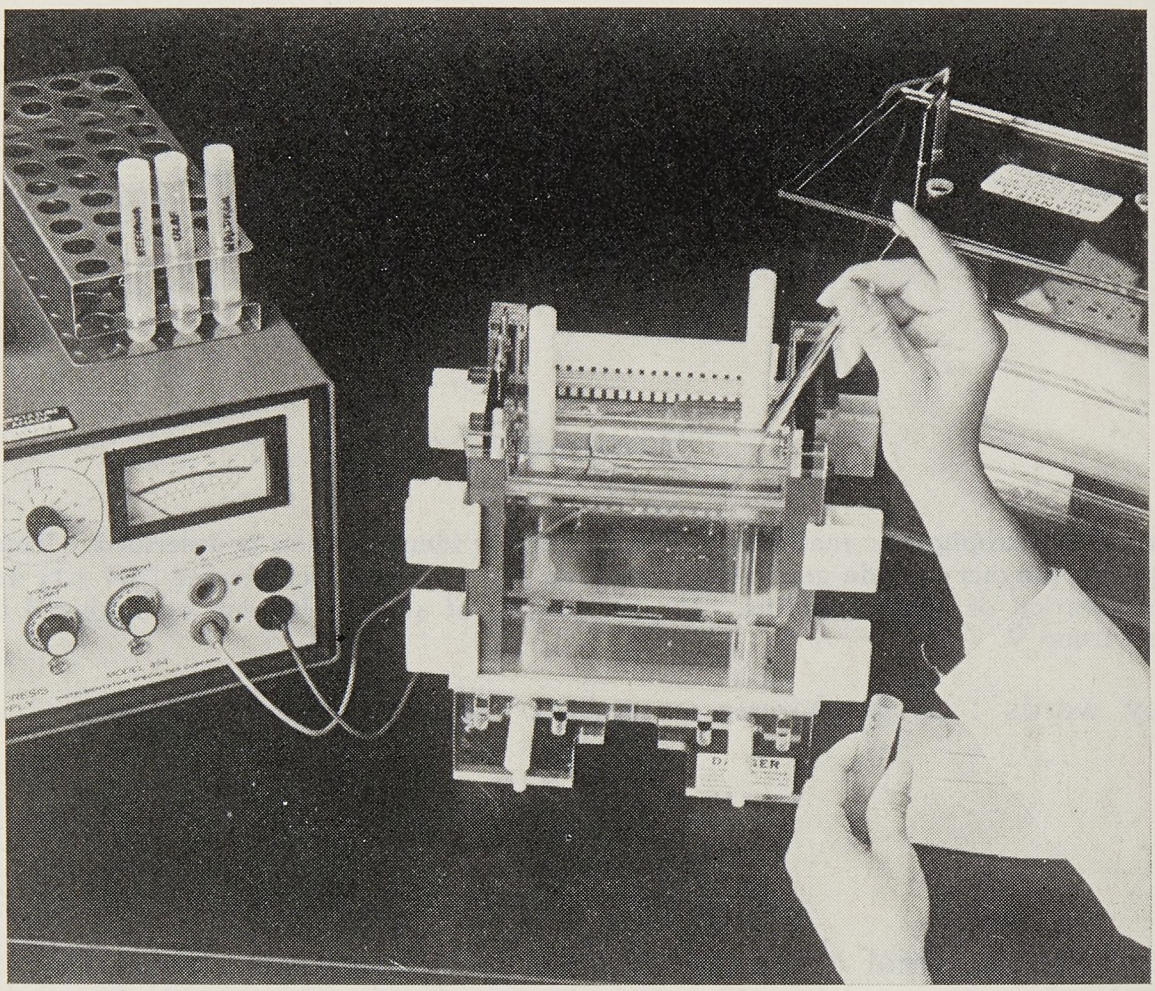

FIG. 1. — *Model 220 dual vertical slab cell for PAGE.*

by circulation of coolant through the inner core of the cell. Either one or two gels may be run at one time. Pre-cast gels may also be used.

The 6 p. 100 gel formulation (BUSHUK and ZILLMAN, 1978) was modified for the vertical cell: 6 g acrylamide, 300 mg N, N-methylene-bisacrylamide, 20 mg ascorbic acid, 2.5 mg ferrous sulphate (heptahydrate) made up to 100 ml with electrophoresis buffer. The buffer used was 0.0085 M aluminium lactate (Fluka AG, Buchs, Switzerland)-lactic acid (pH 3.1) (JONES *et al*, 1959). For polymerization each 0.75 mm gel slab required 15 g gel solution and 60 µl 1 p. 100 hydrogen peroxide. Sample extracts (3 to 5 µl) were applied with microsyringes. Electrophoretic conditions were varied with the number of gels run (one or two) and the gel slab thickness (0.75 or 1.5 mm): for dual 1.5 mm gels, 570 V for 80 min; for a single 0.75 mm gel, 1 400 V for 20 min; and for dual 0.75 mm gels, 1 000 V for 40 min. Circulating coolant was maintained at 7 °C for all runs. After removal from the glass plates, each gel was stained in a solution of 95 ml 12 p. 100

trichloroacetic and 5 ml 0.5 p. 100 Coomassie Brillant Blue R-250 at room temperature, usually overnight for convenience. No destaining solution was required; however, precipitated dye was rinsed from the gel surface with water.

Densitometry

The gel slabs were sliced lengthwise into individual electrophoregrams and each was scanned at 570 nm in a Gilford 2 520 gel scanner (Oberlin, Ohio, U.S.A.).

Results and discussion

To investigate wheat cultivar identification by PAGE, the Model 220 dual vertical slab apparatus was chosen because of certain advantages over other systems: apparatus assembly and gel formation is simple and reproducible, a large number of samples (40) can be electrophoresised at one time, gels are inexpensive (two 0.75 mm gels cost $3 (chemicals and labor) in comparison to $10 for one commercially preformed gel which can accommodate only 14 samples), no pre-electrophoresis is required, and gel slab thickness can be varied.

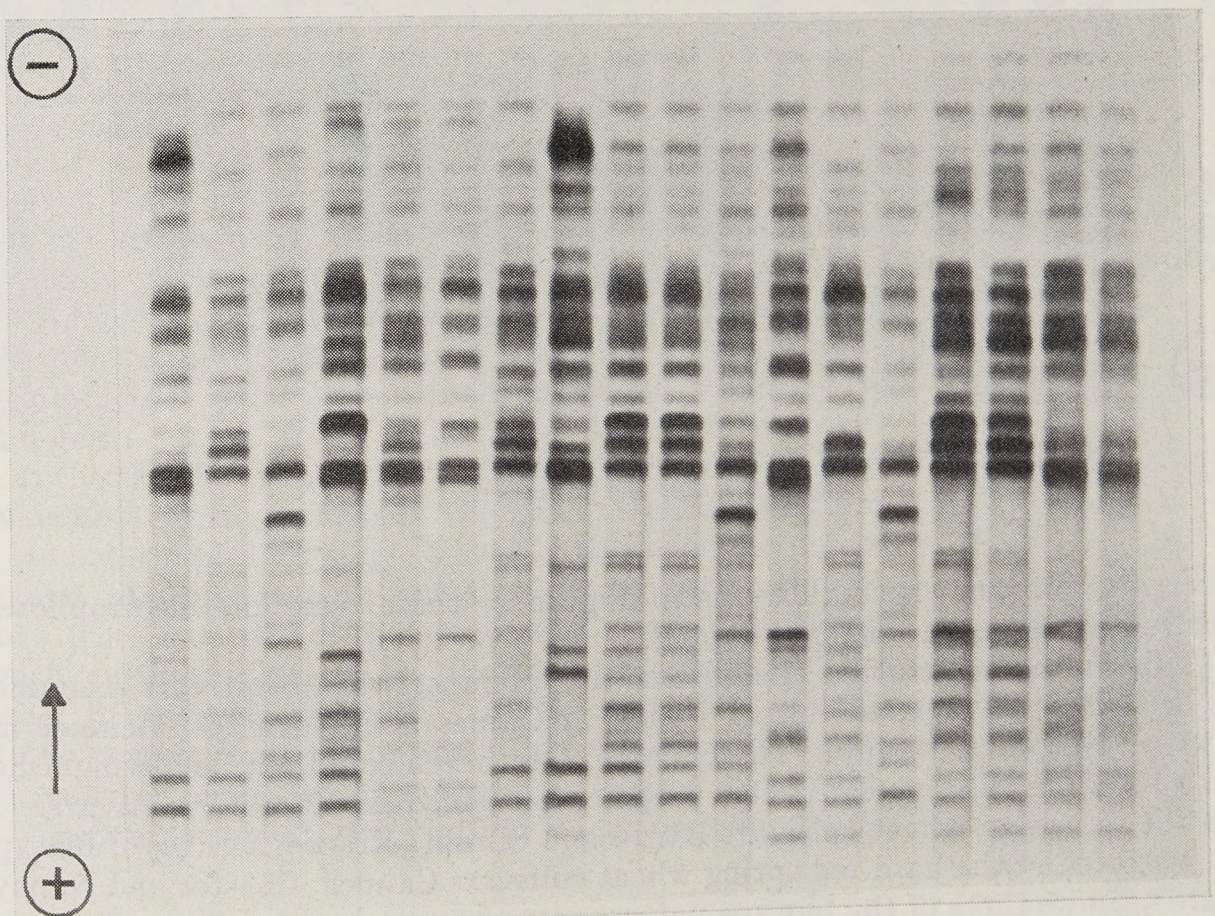

FIG. 2. — *Gliadin electrophoregrams (left to right) for the winter wheat cultivars Fredrick, Gaines, Genesee, Kharkov 22MC, Lennox, Norstar, Nugaines, Sun, Sundance, Sundance (Lethbridge), Talbot, Winalta, Yogo, Yorkstar; the hard red spring cultivars Canthatch, Canuck, Chester (Lethbridge) and Chester (Swift Current). Electrophoresis at 570 V for 80 min with dual 1.5 mm gels.*

To optimize band resolution and electrophoresis time with this system, gel thickness was decreased and run voltage was increased. The gliadin electrophoregrams of some Canadian spring and winter wheat cultivars in Figure 2 resulted from an 80 min run at 570 V with dual 1.5 mm gels. Increasing the voltage to 1 400 V decreased run time to 20 min with a single 0.75 mm gel, but produced electrophoregrams unsuitable for cultivar distinction (Fig. 3). Poor heat dissipation produced blurry, curved patterns, and in addition, caused cracking of the glass gel plates. Since good band resolution was obtained in only 40 min using 1 000 V with dual 0.75 mm gels, as shown for some Canadian spring,

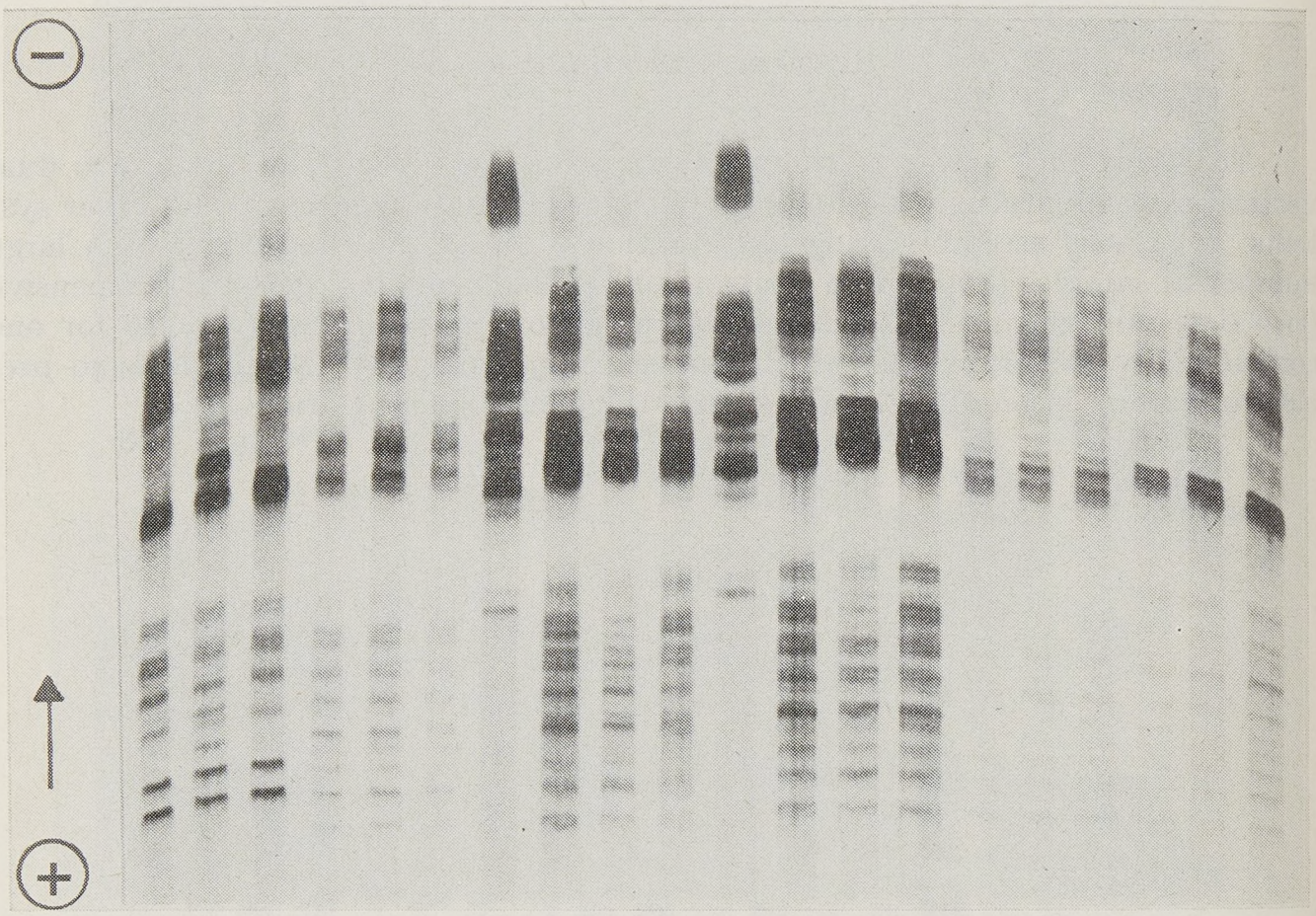

FIG. 3. — *Electrophoregrams from electrophoresis at 1 400 V for 20 min with a single 0.75 mm gel.*

winter and durum wheat cultivars in Figure 4, these were the electrophoretic conditions that were finally adopted.

This method permitted characterization of most wheat cultivars examined; only a few closely related cultivars such as Gaines and Nugaines; Genesee and Yorkstar; Neepawa, Manitou, Thatcher and Canthatch could not be distinguished. This procedure also revealed the presence of biotypes in some cultivars. As with the Canadian wheat cultivars BW20 and Sinton (KOSMOLAK, 1979) biotypes were detected in the hard red spring wheat cultivars Canuck, Chester and Napayo. Figure 5 shows three of the biotype patterns found in 85 breeder lines of the cultivar Canuck. Pattern C, identical to the pattern for the cultivar Canthatch, represents 62 p. 100 of the lines, while patterns B and A, unlike any other catalogued wheat gliadin patterns, represent 31 p. 100 and 2 p. 100 respectively.

The 0.75 mm gels stain rapidly even at room temperature. Since most

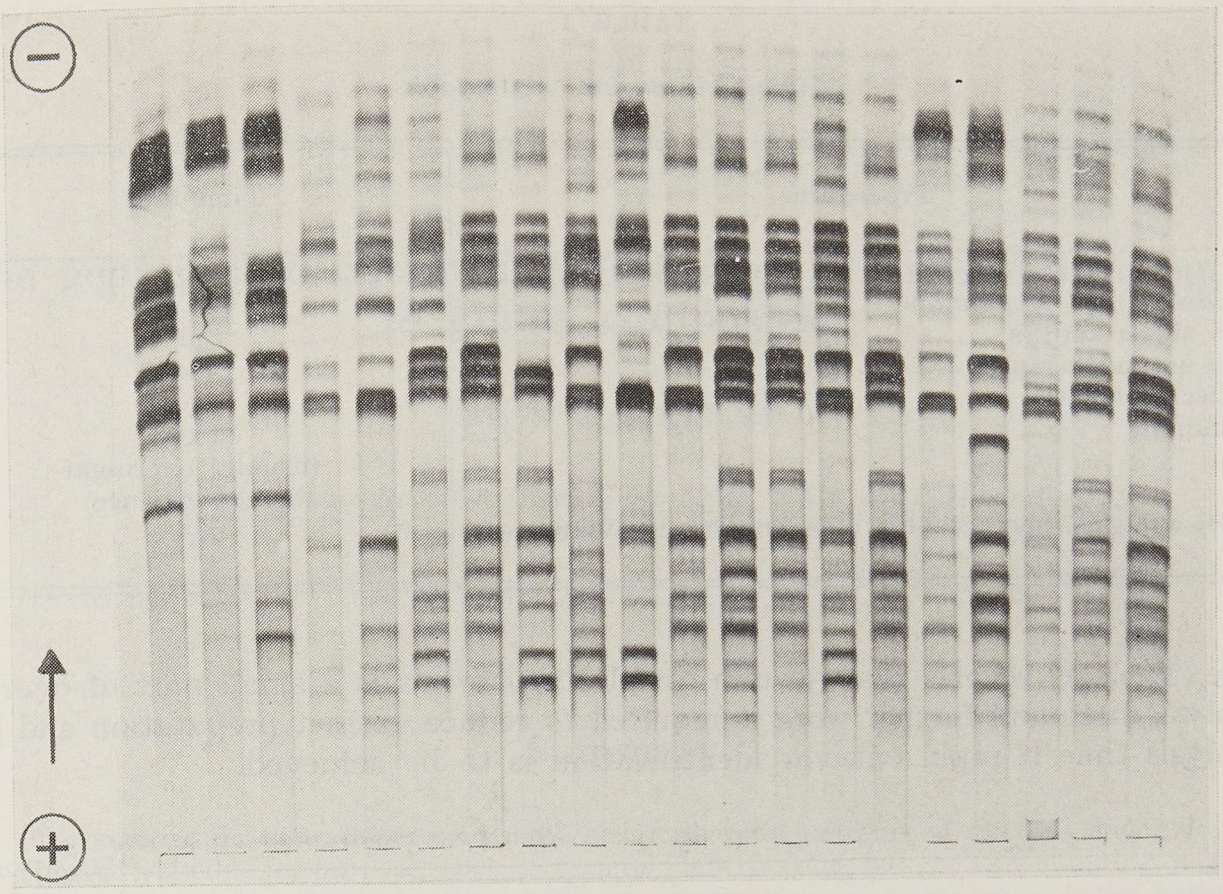

FIG. 4. — *Gliadin electrophoregrams (left to right) for the durum cultivars Wakooma, Pelissier, Macoun; the winter wheat cultivars Norstar, Winalta, Sundance; the spring wheat cultivars Thatcher, Sinton, Selkirk, Pitic 62, Park, Neepawa, Napayo, Marquis, Manitou, Glenlea, Chinook, Chester, Canuk and Canthatch. Electrophoresis at 1 000 V for 40 min with dual 0.75 mm gels.*

bands are visible after 1 h staining, cultivar identification can be made for most cultivars, excepting those that are distinguished by minor bands or quantitative band differences. Also there was a lower relative deviation on the densitometric scans of the electrophoregrams from 0.75 mm gels (7 p. 100) than those from the 3 mm horizontal gels (< 15 p. 100) previously used in the laboratory.

If the sample is known to be pure, its identity can be provided by electrophoresis of the gliadins extracted from one ground sample or a single kernel. However the detection and identification of cultivars in mixtures requires more. The accuracy of the estimation of the proportion of each cultivar present in a mixture depends on the number of kernels examined. In the laboratory usually 40 to 80 kernels from the mixture are analyzed to determine cultivar composition with some degree of reliability. Examination of the cultivar identification timet-

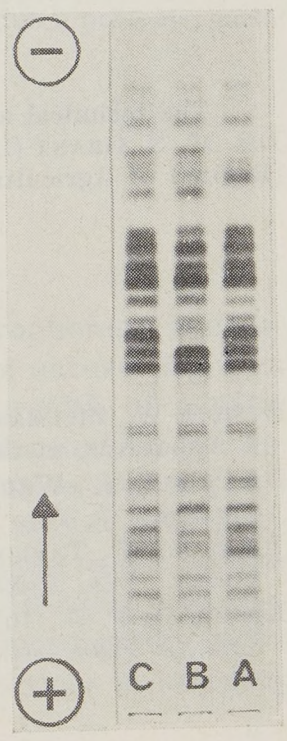

FIG. 5. — *Gliadin electrophoregrams of three biotypes present in the cultivar Canuck.*

TABLE I

Cultivar identification timetable

Procedure	Time-Min
Gliadin extract preparation:	
40 single kernels	150
40 ground samples	300
Electrophoresis	40
Staining	60-120 (usually overnight for convenience)
Gel analysis (photography, densitometric scanning). . .	240

able (Table 1) reveals that 50 min for electrophoresis is a small part of overall analysis time and further work is required to reduce extract preparation and gel analysis time if rapid cultivar identification is to be achieved.

Reçu pour publication en octobre 1980.

Résumé

Une méthode électrophorétique rapide pour l'identification des variétés de blé est décrite. Des gels minces (0,75 nm) à 6 p. 100 de polyacrylamide en tampon lactate d'aluminium (pH 3,1) soit utilisés pour le fractionnement des gliadines du blé.

L'électrophorèse de 40 échantillons peut être réalisée en seulement 40 minutes à 1 000 V.

Acknowledgements

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The genetic and chemical significance of varietal differences in gluten composition

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(to be presented by Dr Ian Batey)

Summary

The many differences observed in the electrophoretic patterns of gliadins and glutenins have proved valuable for purposes such as varietal identification, but the long-standing question of their possible relationship to quality largely remains unanswered. One reason is the limitation imposed by the complex nature of both quality and gluten composition. To overcome this problem, we have devised computer-based methods of pattern analysis to permit the comparison of a large number of genotypes (80) according to electrophoregram and a range of quality (and other) attributes.

Firstly, the procedures provide a basis for computerized identification of cereal varieties, and for the preparation of an identication key. Secondly, the analyses show that certain protein components are often associated as "blocks" of bands, presumably equivalent to the groups identified by crossing experiments to be due to the clustering of tightly linked genes.

In addition, we have examined the frequency with which individual gliadin or glutenin components are associated with a range of characteristics including grain hardness and resistance of dough to extension. Certain bands have been identified as being correlated with specific aspects of quality. Such associations are partly due to pedigree relationships in the set of varieties examined, but others may be due to chemical differences between the gluten proteins, particularly in their sulphur content.

Key words : *gluten proteins — electrophoresis — genetics — quality.*

During the past two decades, techniques such as gel electrophoresis and isoelectric focusing have progressively revealed to us the complex nature of gluten protein. These techniques have also provided a key to the wealth of genetic information locked up in the gluten proteins. Electrophoretic analysis of the gliadin proteins, particularly, has been used to provide information about genotype for the purposes of identifying cultivars (Ellis, 1971; Wrigley and Shepherd, 1974; Autran and Bourdet, 1975), for distinguishing between species and between genera in food analysis (Wrigley, 1977), for checking off-types encountered in pure-seed production (Appleyard *et al.*, for investigating pedigrees (Wrigley and Shepherd, 1977), and for a range of applications in breeding studies.

Identification of wheat cultivars

In fact, gliadin electrophoresis is a good example of the application of a pure research project, originally designed solely to investigate the composition of gluten. From these esoteric beginnings, it has developed into a range of routine tests that have made " electrophoresis " a household word for wheat growers (WRIGLEY, 1980). In Australia, starch gel electrophoresis has served us well for varietal identification in the mid-1970's, but in recent years gradient gel electrophoresis (MARGOLIS and WRIGLEY, 1975; DU CROS and WRIGLEY, 1979) has replaced it due to its greater speed, convenience and resolution. It is the

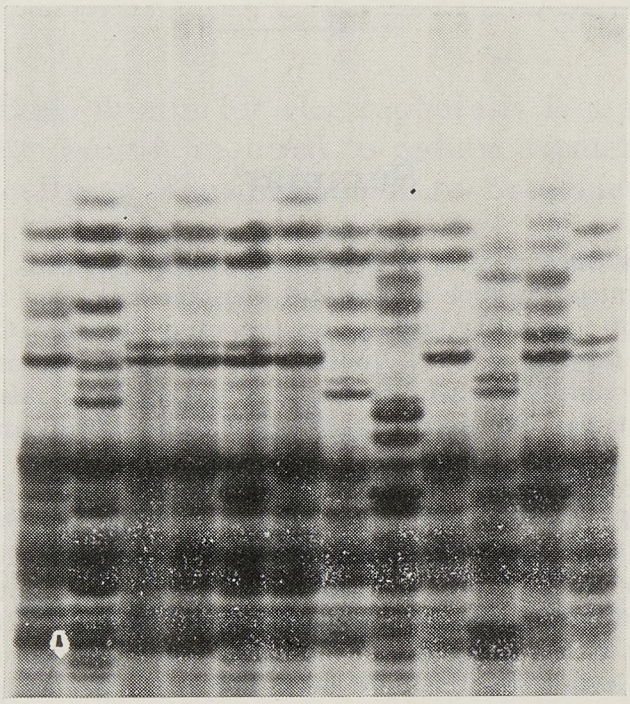

FIG. 1. — *Gradient gel electrophoresis of gliadin proteins from the cultivars (left to right) Olympic, Millewa, Gamenya, Warimba Lance, Condor, Tincurrin, Halberd, Timgalen, Durati, Eagle and Cook. Polyacrylamide concentrations increase from 2.5 p. 100 (top, origin, anode) to 13 p. 100 at the bottom of the preformed gel (Gradient Pty Ltd, Lane Cove, 2066, Australia). Electrophoresis was performed at 200 V for 2 h as described by* DU CROS *and* WRIGLEY *(1979).*

theme of a poster, produced by the Australian Wheat Board, to publicize to farmers the ability of its laboratories to positively identify the wheat delivered. Figure 1 shows gradient-gel electrophoregrams for some Australian wheats.

Most of these studies on genotypic characterization have been based on gliadin composition. However the glutenin fraction, though until recently more difficult to fractionate, holds further valuable information. SDS electrophoresis in a 7 to 16 p. 100 gradient of polyacrylamide provides a ready means of analysing glutenin subunit composition. Figure 2 shows how this method has proved of practical value in distinguishing between some closely related wheat cultivars that are not readily distinguishable by gliadin electrophoresis.

Protein composition and grain quality

In contrast to these successful applications of gluten-protein fractionation, one of the original aims of such studies has not been realised: we do not understand the relationship, if any exists, between grain quality and gluten-protein composition. There have been many attempts to correlate composition and

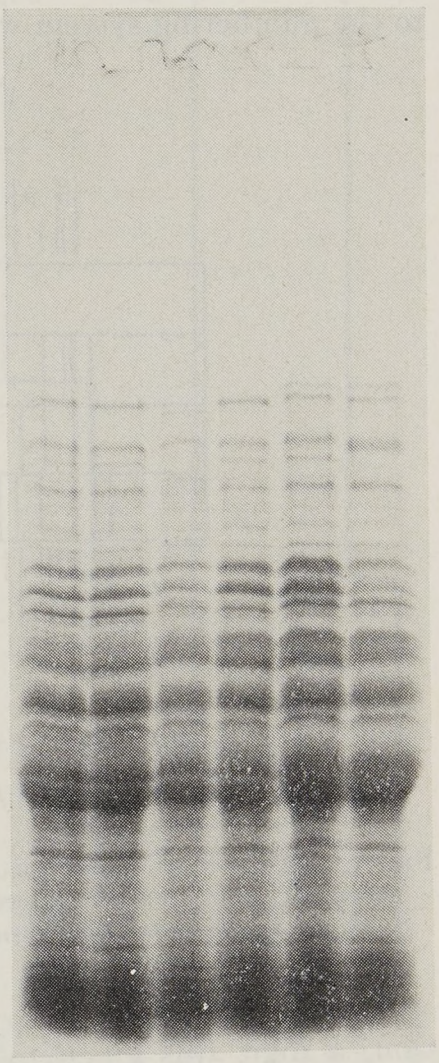

FIG. 2. — *DSD electrophoresis of endosperm proteins, extracted with 4 p. 100 SDS - 1.5 p. 100 mercaptoethanol-Tris buffer, pH 6.8. The gel contained a gradient of polyacrylamide from 7.5 p. 100 at the top (origin, cathode) to 16 p. 100 at the bottom. The buffer system was a modification* (LAWRENCE *and* SHEPHERD, 1979) *of that described by* KING *and* LAEMMLI (1971). *From the left, cultivars represented are Condor B, Condor A, Egret, Oxley, Shortim and Timson. Distinctions between some of these closely related wheats (Condor vs Egret; Shortim vs Timson) are provided in the patterns for the glutenin subunits (top quarter of electrophoregram).*

quality (e.g. ELTON and EWART, 1960; COULSON and SIM, 1964; HUEBNER and ROTHFUS, 1968; DOEKES, 1968; HUEBNER, 1970; ORTH and BUSHUK, 1972 and 1973; GUNZEL, 1976). To expect such a relationship appears reasonable. Varying degrees of success have been claimed, but no such association is generally accepted.

Because of the complex nature of both factors, quality and protein composition, any comparisons have been restricted to relatively small numbers of genotypes. To overcome this limitation, we developed a series of computer-based programmes, in association with Drs W.T. Williams and P.J. Robinson of CSIRO Division of Tropical Crops and Pastures, Townsville. Our aim was to apply methods of pattern analysis to the comparison of large numbers of wheat genotypes (WILLIAMS, 1976).

Systematic identification of cultivars

An initial aim of these studies was to devise a computer-based system of electrophoregram comparison for identification of wheat cultivars. Electrophoretic patterns were first reduced to a series of numbers to facilitate systematic comparison. Sufficient band positions were allocated to provide for all bands in the patterns being considered. These positions are numbered 1 to 31 in Figure 3. Later consideration of other cultivars necessitated the allocation of band position 32. Bands 33 and 34, which did not vary for any of the wheats, were added to the numbering system when rye was considered. Secondly, for each band posi-

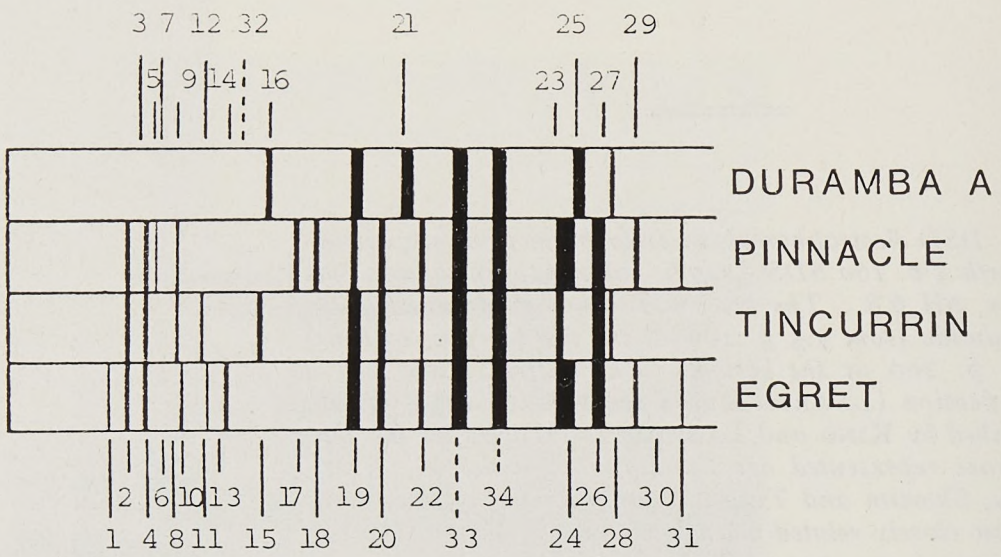

FIG. 3. — *Diagrams of electrophoretic patterns and gliadin band positions used for computer-based comparisons (from* WRIGLEY *and* MC CAUSLAND, 1977). *The electrophoregram of Egret is shown in the numerical form used for computer-based comparison.*

tion, a digit was given to indicate band intensity. Absence was shown as 1; light, medium and dark intensities as 2, 3 and 4.

The conversion of electrophoretic patterns to numerical form involved some simplification and interpretation. Data from both starch and gradient gels were used, but most reliance was placed on the former. Some extra components resolved by the latter technique were not included. In other cases, components of similar mobility were indicated as a single component although resolution into more than one band was apparent. In fact, two-dimensional fractionation has shown that many electrophoretic components can be further subfractionated by gel isoelectric focusing (WRIGLEY, 1970).

The method of comparison used (MULCLAS, part of the TAXON package on the Cyber 76 computer, CSIRO Camberra) made it possible to compare the electrophoregram of a sample of unknown identity with those of a range of cultivars. A limited selection of varietal patterns were then provided by the programme, each pattern being accompanied by a quantitative indication of its similarity to

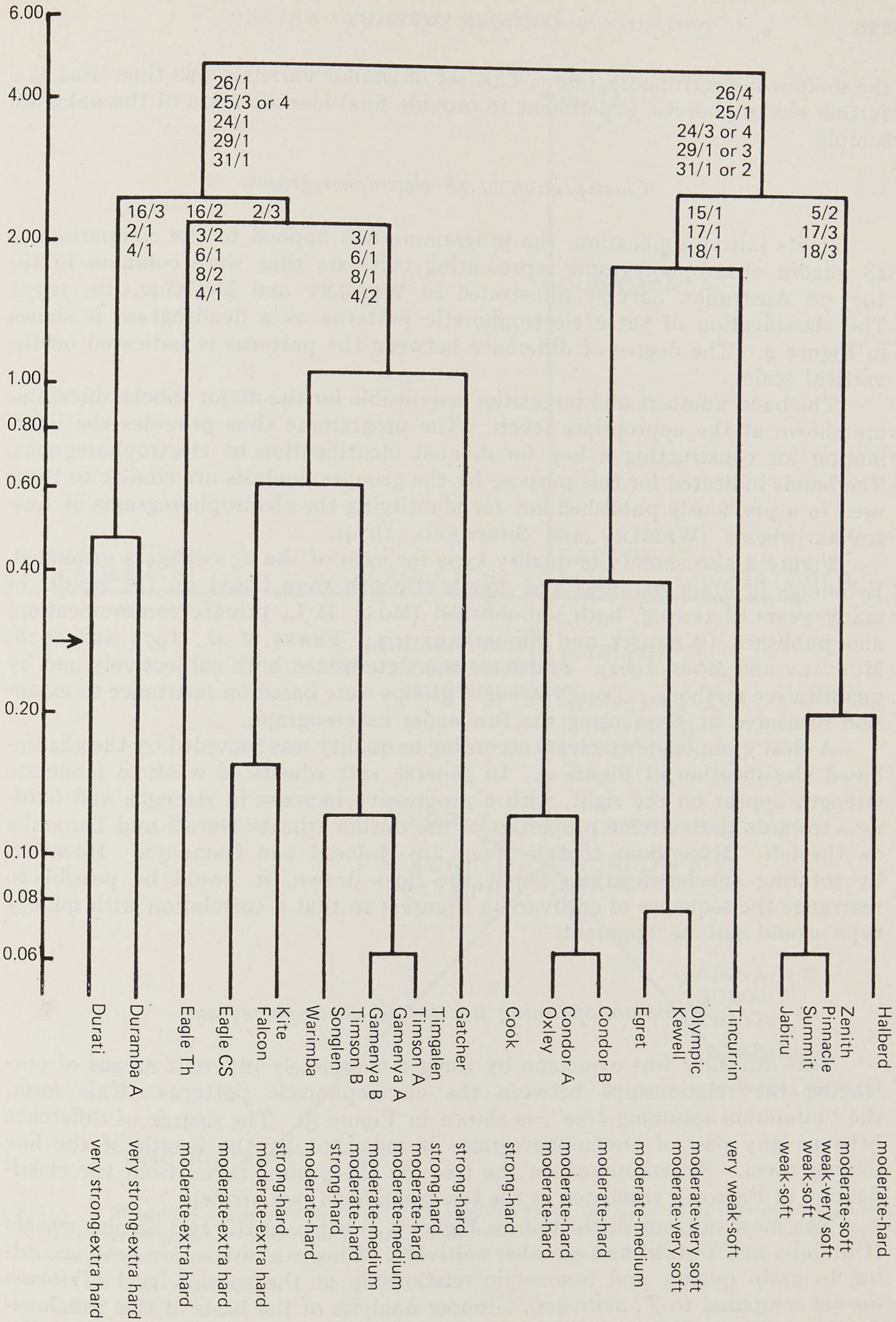

FIG. 4. — *Classification of the gliadin electrophoregrams of wheats currently grown in Australia. Differences between patterns are indicated quantitatively on the log scale at left. The band numbers and intensities (e.g. 26/4 - band 26, strong) used in making the major subdivisions are shown. Quality type is indicated for each cultivar as a combination of dough strength and grain hardness.*

the unknown electrophoregram. This set of similar varieties was then used in a further electrophoretic experiment to provide final identification of the unknown sample.

Classification of 18 electrophoregrams

In its initial application, the programme was applied to the comparison of 18 gliadin electrophoregrams representing cultivars that were common in the 1977-78 Australian harvest (illustrated in WRIGLEY and MCCAUSLAND, 1977). The classification of these electrophoretic patterns as a dendrogram is shown in Figure 4. The degree of difference between the patterns is indicated on the vertical scale.

The band numbers and intensities responsible for the major subclassifications are shown at the appropriate levels. The programme thus provides the information for constructing a key for manual identification of electrophoregrams. The bands indicated for this purpose by the grouper analysis are similar to those used in a previously published key for identifying the electrophoregrams of Australian wheats (WRIGLEY and SHEPHERD, 1974).

Figure 4 also shows the quality type for each of the 23 cultivars examined. Estimates of grain hardness and dough strength were based on the results of many years of testing, both unpublished (MOSS, H.J., private communication) and published (WRIGLEY and SHEPHERD, 1974; FERNS *et al.*, 1975 and 1978; MULLALY and MOSS, 1961). Hardness was determined both subjectively and by quantitative methods. Dough strenght groups were based on resistance to extension measured at 5 cm using the Brabender extensograph.

A clear grouping of cultivars according to quality was provided by the gliadin-based classification of Figure 4. In general, soft wheats of weak to moderate strength appear on the right, with a progressive increase in strength and hardness towards the extreme properties of the durum wheats Durati and Duramba on the left. Exceptions to this trend are Halberd and Gamenya. However, by rotating subclassifications about the lines drawn, it would be possible to rearrange the sequence of cultivars in Figure 4 so that a correlation with quality type would not be apparent.

Minimum spanning tree for 18 electrophoregrams

This difficulty was overcome by using a completely different means of portraying the relationships between the electrophoretic patterns. This form, the "minimum spanning tree", is shown in Figure 5. The degree of difference between any pair of electrophoregrams is indicated by the length of the line joining them. Superimposed on the tree are the groups taken from the classification of Figure 4, truncated at the ten-group level (see arrow).

The most obvious distinction in Figure 5 is between the two durum wheats (Duramba and Durati) and all other cultivars. This is a distinction both according to grain quality and taxonomic relationship at the species level (*Triticum durum* compared to *T. aestivum*). Grouper analysis of the basis of this subclassification indicates that it is largely due to the durums' lack of low-mobility gliadins (positions 1 to 6). As their synthesis is probably controlled by genes on chromosome 1D (WRIGLEY and SHEPHERD 1973), the basis of this distinction is primarily genetic.

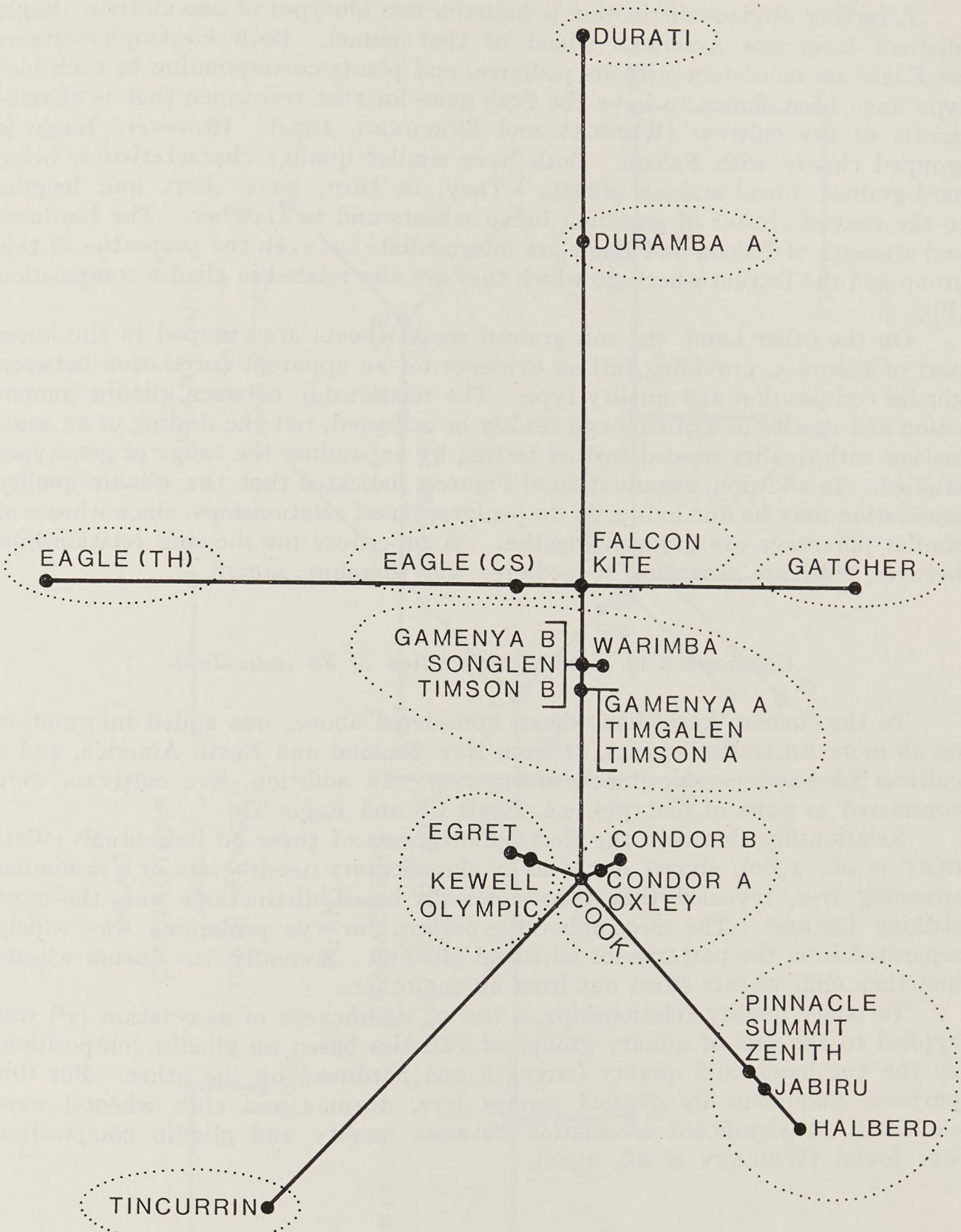

FIG. 5. — *Minimum spanning tree showing, in a different way, the relationships between the gliadin electrophoregrams referred to in Figure 4 for current Australian wheat cultivars. Circles indicate groups of varieties classified in Figure 4 at the ten-group level.*

A second obvious distinction in Figure 5 is the separation of the soft weak club-wheat Tincurrin. This also has a taxomic basis, since a separate sub-species (*compactum*) has been allocated to distinguish club from common wheat (BRIGGLE and REITZ, 1963).

A further obvious distinction is between two biotypes of one cultivar, Eagle (distinct from the American wheat of that name). Both electrophoregrams for Eagle are consistent with its pedigree, and plants corresponding to each biotype have been shown to have the Sr26 gene for rust resistance that is characteristic of the cultivar (WRIGLEY and SNEPHERD, 1974). However, Eagle is grouped closely with Falcon. Both have similar quality characteristics, being hard-grained bread-making wheats. They, in turn, have short link lengths to the central cluster of premium bread wheats and to Gatcher. The hardness and strength of Falcon and Eagle are intermediate between the properties of this group and the Durum wheats to which they are also related in gliadin composition (Fig. 5).

On the other hand, the soft-grained weak wheats are grouped in the lower part of Figure 5, providing further evidence for an apparent correlation between gliadin composition and quality type. The relationship between gliadin composition and species in Triticum can readily be accepted, but the finding of an association with quality needed further testing by expanding the range of genotypes studied. In addition, examination of Figure 5 indicated that the gliadin-quality association may be due indirectly to pedigree-based relationships, since wheats of similar parentage are grouped together. A procedure for showing relationships between cultivars according to pedigree was therefore sought.

Comparison of gliadin composition of 80 individuals

To the current Australian wheats considered above, was added information on 40 more Australian wheats, 11 from New Zealand and North America, and a cultivar of *Secale cereale* (black winter rye). In addition, five cultivars were considered as pairs of biotypes, e.g. Eagle CS and Eagle Th.

Relationships between the electrophoregrams of these 80 individuals (WRIGLEY *et al.*, 1980), shown either as a classificatory dendrogram or a minimum spanning tree, revealed that taxo nomically-based distinctions were the most striking features. The electrophoretic pattern for rye prolamins was widely separated from the patterns for all wheat gliadins. Secondly, the durum wheats and then club wheats stood out from all the others.

To study quality relationships, a test of significance of association (χ^2) was applied to the sets of affinity groups of varieties based on gliadin composition, on the one hand, and quality (strength and hardness) on the other. For this purpose, taxonomically distinct groups (rye, durums and club wheats) were excluded. A significant association between quality and gliadin composition was found (WRIGLEY *et al.*, 1980).

Inverse pattern analysis

Having found that this relationship was still evident in the expanded set of genotypes, the next step was to determine the specific gliadin bands associated with various aspects of quality. Inverse pattern analysis was used for this purpose; the relationships are shown in the form of a minimum spanning tree in Figure 6. To further illustrate the use of this type of analysis, morphological descriptions of plants, heads and grains were included from books on visual identification of about 60 Australian wheats (FERNS *et al.*, 1975 and 1978).

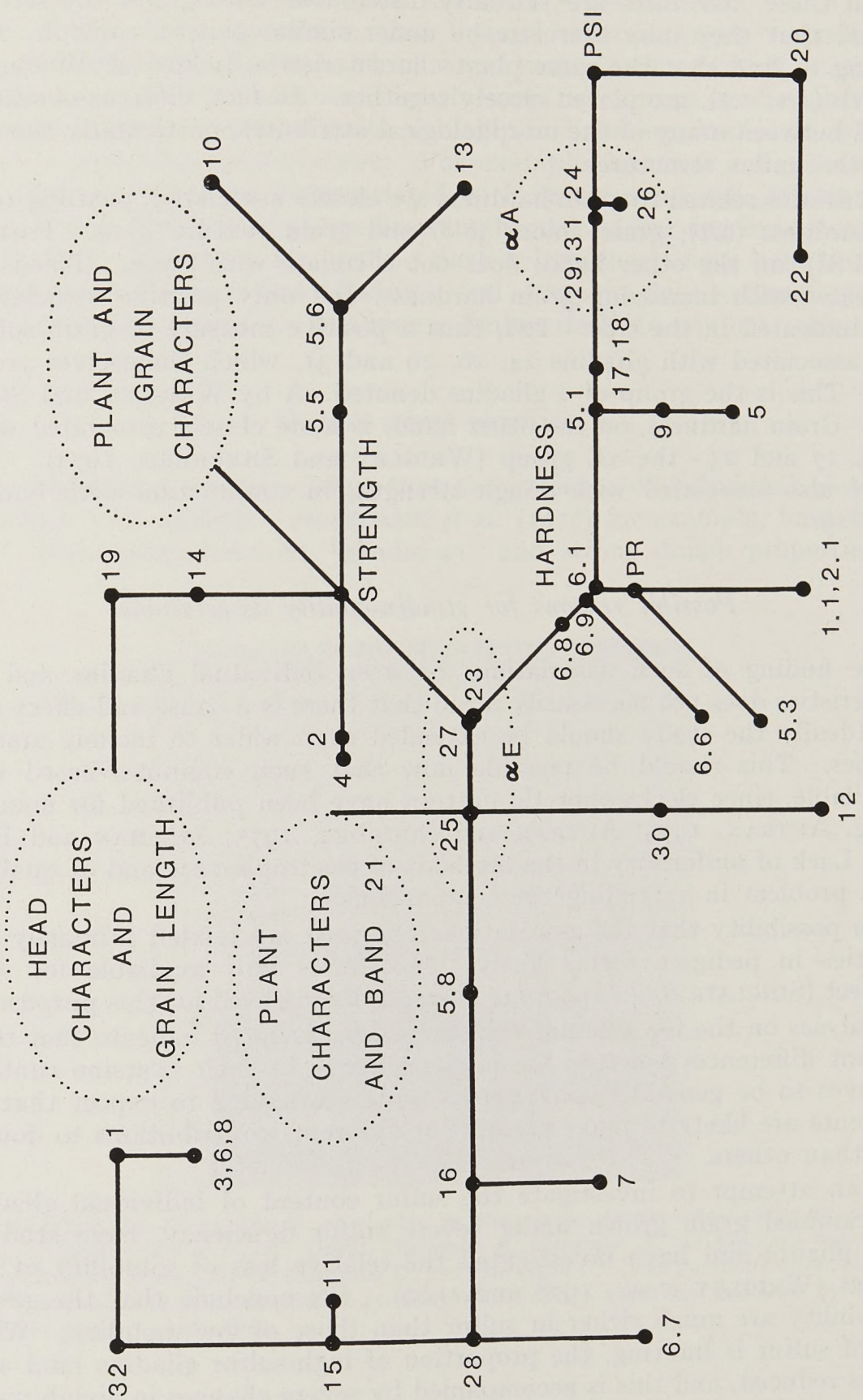

FIG. 6. — *Minimum spanning tree showing relationships between gliadin bands (1 to 32 as in Fig.3), dough strength, pearling resistance (PR), particle size index (PSI), and characters used to describe plants, heads and grain in* FERNS *et al. (1975 and 1978). The characters specifically shown are 1.1 and 2.1-auricle hairiness in plants at 3-6 and 6-8 leaf stages, 5.1-glume colour, 5.3-shape of head in cross-section, 5.8-glume shoulder shape, 6.1-grain hardness, 6.6-germ width, 6.7-height/width ratio for grain, 6.8-grain colour, 6.9-grain texture. Cultivars considered were the 60 cultivars described by* FERNS *et al. (1975 and 1978).*

The location of two attributes close to one another in the tree (Fig. 6) indicates that these attributes are similarly distributed throughout the set of cultivars, and that they may therefore be under similar genetic control. It is not surprising to find that the same plant characteristics, judged at different stages of growth (1.1, 2.1), are placed closely together. In fact, close associations were revealed between many of the morphological attributes, particularly those associated with similar structures.

Attributes related to grain hardness are closely associated: pearling resistance (PR), hardness (6.1), grain colour (6.8) and grain texture (6.9). Particle size index (PSI), on the other hand, does not correlate with these. This is because it decreases with increasing grain hardness, and only positive associations are clearly indicated in the tree. PSI, thus a positive measure of grain softness, is closely associated with gliadins 24, 26, 29 and 31, which themselves are closely linked. This is the group of α-gliadins denoted αA by WRIGLEY and SHEPHERD (1974). Grain hardness, on the other hand, is more closely associated with gliadins 23, 27 and 25 - the αE group (WRIGLEY and SHEPHERD, 1974). These in turn are also associated with dough strength, in conjunction with bands 2, 4, 14 and 19.

Possible reasons for gliadin-quality associations

The finding of such associations between individual gliadins and quality characteristics does not necessarily mean that there is a cause-and-effect relationship. Ideally the study should be extended even wider to include many more genotypes. This should be possible now that such computer-based methods are available, since electrophoretic pattern have been published for many varieties (e.g. AUTRAN, 1975; AUTRAN and BOURDET, 1975; ZILLMAN and BUSHUK, 1979). Lack of uniformity in the methods of electrophoresis and of quality testing is a problem in extending the comparisons.

The possibility that the associations observed are related primarily through similarities in pedigree seems likely. Procedures now available for studying this aspect (SINCLAIR *et al.*, 1980) are currently being used for this purpose.

Analyses on the few gliadins that have been isolated indicate that there are significant differences between them, particularly in their cysteine content. If this proves to be generally so, it seems quite reasonable to expect that certain components are likely to make greater (or different) contributions to dough properties than others.

In an attempt to investigate the sulfur content of individual gliadins, we have examined grain grown under severe sulfur deficiency, have studied ^{35}S-labelled gliadin and have investigated the relative loss of solubility of gliadins with heat (WRIGLEY *et al.*, 1978 and 1980). We conclude that the gliadins of high mobility are much richer in sulfur than those of low mobility. When the supply of sulfur is limiting, the proportion of high-sulfur gliadins (and also glutenins) is reduced, and this is accompanied by severe changes in dough properties (increased resistance to extension and decreased extensibility) (MOSS *et al.*, 1980). Presumably then, genotypic differences in the proportions of high- and low-sulfur proteins could partly explain differences between cultivars in dough properties.

Such explanations for inter-varietal differences in grain hardness seem less reasonable since, for example, isogenic lines of very different hardness have been shown to be identical in gliadin composition (SIMMONDS *et al.*, 1973). Howe-

ver, our knowledge of the biochemical nature of grain hardness is so scant that we are hardly in any position to make satisfactory predictions.

Alternatively, there may be a close linkage between genes controlling the synthesis of individual gliadins and of important quality factors. Although, in such a case, no direct biochemical involvement is inferred on the part of the gliadins, such findings could be quite valuable in breeding studies to select for progeny of a particular quality type. For example, bands 2 and 4 have been shown in Chinese Spring to be associated with chromosome 1D (WRIGLEY and SHEPHERD, 1973), which in turn has been implicated as carrying important genes for flour quality (MORRIS *et al.*, 1978). Such a hypothesis may help to explain the observation in Figure 6 that gliadins 2 and 4 appear close to dough strength, but the weak dough properties of Chinese Spring itself do not bear this out.

Durum wheat gliadins

There have been various claims that the presence of individual gluten proteins correlate with quality. DAMIDAUX *et al.* (1978) for example, have found a consistent relationship between "gliadin-45" and strong dough properties whe-

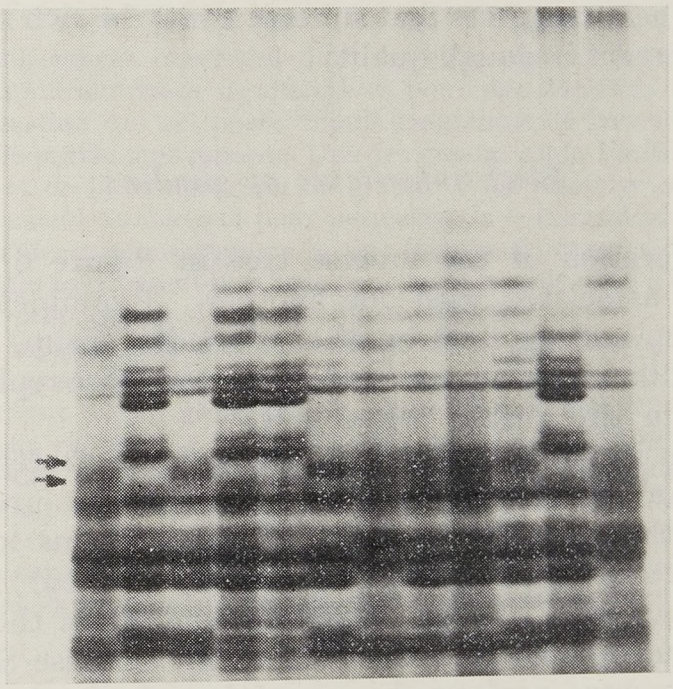

FIG. 7. — *Gradient gel electrophoresis of gliadin proteins from durum-wheat cultivars and lines bred in Australia (left to right) : Dural, Duramba B, Duramba A, six breeder's lines selected for good dough quality, Durati, Duramba D, and Duramba C. The positions of the electrophoregrams equivalent to gliadins-42 and-45* (DAMIDAUX *et al.*, 1978) *are indicated by the upper and lower arrows, respectively.*

reas poorer quality was associated with band 42. In collaboration with Dr Autran (Montpellier), our three Australian durum cultivars were examined. All have strong dough properties, and all have "gliadin-45", equivalent to the region of our band 19 (Fig. 7), which itself is shown to be associated with strength in Figure 6. More recently, we examined six breeding lines, selected by Dr Hare (Agricultural Research Centre, Tamworth, NSW) to have strong dough properties. Four of

these had the "quality-positive" gliadin (Fig. 6), but two of them had gliadin-42 (in the region of our bands 17 and 18) instead of gliadin-45.

The four biotypes of the Australian durum cultivar Duramba may provide an opportunity for testing the proportion. Biotype A predominantes in commercially grown crops, but three other lines have been identified having different electrophoregrams but otherwise similar characteristics. Durambas A and C have gliadin-45; biotypes B and D have gliadin-42 (Fig. 7). They thus constitute a set of isogenic lines in which to study the relationship between these gliadin components and quality.

Glutenin proteins

Glutenin subunit 1 is another recent example of an apparent positive correllation between a gluten protein and grain quality (PAYNE *et al.*, 1979). In collaboration with Drs Lawrence and Shepherd (Waite Agricultural Research Institute, Adelaide, South Australia), we are comparing quality, gliadin composition, and pedigree characteristics (for the full set of wheats mentioned above) with their glutenin-subunit composition as revealed by SDS electrophoresis (LAWRENCE and SHEPHERD, 1979). According to preliminary indications, subunit 1 is not closely associated with quality in these wheats. On the other hand, our study of sulfur-deficient wheats suggests that subunit 1 may be rich in sulfur and possibly for that reason important in dough quality.

Block inheritance of gliadins

Finally, consideration of the inverse tree in Figure 6 shows that certain gliadins are closely associated with one another. Examples already mentioned are bands 24, 26, 29 and 31, and another α-gliadin group, 23, 25 and 27. In the ω-gliadin region, bands 2 and 4 occur together, and are replaced in some wheats where all gliadins in this region are absent.

These and other examples suggest that the gliadin proteins occur in the electrophoregrams as groups of components that are either present as a whole or absent. Study of the inheritance of the gliadin proteins has produced results pointing to similar conclusions (QUALSET and WRIGLEY, 1977; SOZINOV and POPERELYA, 1978; MECHAM *et al.*, 1978). Gliadin bands from the cultivar Cheyenne were found to be substituted into the pattern for Chinese Spring when electrotrophoretic analyses were performed on certain substitution lines of Cheyenne in Chinese Spring. The gliadins in these groups (or blocks) remained togetheras units when one substitution line was crossed with another. Statistical analyses on the segregation ratios from nearly 400 F2 grains indicated that allelic loci govern the blocks of bands contributed by either parent (QUALSET and WRIGLEY, 1977). Investigation of the subunits of the glutenin proteins suggest that they too occur as blocks of components (LAWRENCE and SHEPHERD, 1979).

Our current interest is to examine the extent to which there is chemical similarity between the components of such iso- or allelic loci. Investigations so far provide support for Dr Sozinov's suggestion that the genetic control of the gliadin proteins in blocks should form the basis for a system of nomenclature. I would go further to suggest that such a system might also be extended to the subunits of the glutenin class of protein.

Presumably these blocks of ,,isogliadins'' and ''allelic gliadins'' (and glutenins) reflect the progressive derivation of our present set of genes for storage-protein synthesis from a much simpler one in the ancient progenitors of today's wheats. Mutations and duplication occured, largely unchecked, to produce clusters of iso-loci, in similar positions to allelic loci which in other genotypes gave slightly different gene products. Thus we have the polymorphism, now evident in the storage protein of today's wheats. However, we may not be correct in our assumption that thepolymorphism arose due to lack of selection pressure against it. Possibly it conferred specific advantages on the plant. It certainly provides us with useful information about the present and past genetic constitution of the plant. Furthermore, as we learn more about the chemistry of the individual storage proteins, we may also see how they contribute to the unique bread-making qualities of wheat in general and of certain cultivars in particular.

Reçu pour publication en octobre 1980.

Résumé

Signification génétique et chimique des différences variétales dans la composition du gluten

Les nombreuses différences observées dans les diagrammes électrophorétiques des gliadines et gluténines se sont montrées significatives pour des odjectifs tels que l'identification des variétés, mais la question qui est posée depuis longtemps de leur relation possible quant à la qualité, reste en grande partie sans réponse. Une des raisons est la limite imposée par la nature complexe de la qualité et de la composition du gluten. Pour résoudre ce problème, nous avons établi des méthodes de calcul numérique pour permettre la comparaison d'un grand nombre de génotypes (80) d'après l'électrophorégramme et une gamme de critères de qualité (et autres).

D'adord, ces procédés fournissent une base pour une identification à l'ordinateur des variétés de céréales, et pour la préparation d'une clé d'identification.

Ensuite, l'analyse montre que certains constituants protéïniques sont souvent associés par groupes de bandes, probablement équivalents aux groupes identifiés par des expériences de croisement comme étant dû au groupage de gênes étroitement liés.

En outre, nous avons examiné la fréquence avec laquelle des composants de la gliadine ou glutenine individuelles sont associées avec une gamme de caractéristiques, tel que la dureté du grain et la résistance de la pâte à l'extension. Certaines bandes ont été identifiées comme étant en correlation avec des aspects spécifiques de la quantité. De telles associations sont en partie dues à la relation de parenté dans l'ensemble des variétés étudiées, mais d'autres peuvent être dues à des différences chimiques entre les protéines du gluten notamment dans leur contenu soufré.

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Genetic classification of prolamines and its use for plant breeding

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Summary

Gliadins proteins were examined from seeds of the F_1, F_2, F_3, F_4 progeny of different crossings by starch gel electrophoresis in vertical glass tubes.

New hybrid proteins are not formed in F_1 and gliadin composition indicates that components are inharited according to the codominant type. Analysis of F_2 seeds enables to shape a concept of blocks of componants which are inharited in a linkage form and controlled by allelic loci. Monosomic analysis allows to determine the chromosomic location of genes coding for these gliadin blocks and to build a block nomenclature.

A such genetic classification of storage proteins is completely different from the existing biochemical classification. Blocks are phenotypical markers of polygenic loci at molecular level so that this genetic classification enables to obtain information on the characteristics genotypes.

The accumulated data also suggest that prolamine allelic blocks are markers of gene clusters or non recombinant parts of chromosomes that play an important role in the formation of some characters like technological qualities of grain, low temperature tolerance, spike colour and discase resistance. The same should be found in other crops.

Although the relationships between allelic blocks and variation in traits are not quite clear the electrophoretic fractionation of gliadin is widely used in wheat and barley area and will help to solve many problems of plant breeding and seed production.

Key words : *prolamines — Plant breeding — gliadins — proteins — electrophoresis.*

In recent years the amount of the works devoted to the study of genetically determined polymorphism of storage proteins has considerably increased. The use of starch and later of polyacrylamide gels with added urea and aluminium lactate buffer, made it possible to separate prolamines into several components (18, 21). Soon it was found that the protein electrophoretic profile was determined by a genotype and practically did not vary with growing conditions (14, 16). It ena-

bled us to offer the methods for the identification of wheat cultivars by comparing patterns (13) or by recording cultivar formulae based on the comparison with the reference profile made up of the three probable precursors of genomes A, B and D (I). French, Canadian and Australian workers (12, 13, 22) developed simpler gliadin nomenclature systems based on fixing the components' mobility in relation to the band common for all the cultivars. All these methods are based entirely on the biochemical principles of protein identification according to their mobility in gels.

The most important stage in the studies on a genetic nature of storage proteins polymorphism was the establishment of the chromosomes of homocologous groups I and 6, crucial for the synthesis of these proteins (20). Regularities in the inheritance of prolamine components were studied as well (15, 6, 19).

At present many papers in the periodicals of different countries are devoted to polymorphism studies of prolamines in wheat, barley, maize and other cereals. Protein identification serves as the basis for the improvement of a genome composition of cultivated and wild cereal species, it allows the development of means of studying heterogeneity of cultivars (7) and yields the data on a genetic control of prolamine biosynthesis in barley, maize, sorghum, etc. Unfortunately, in all these studies it was impossible to establish a distinct correlation between the composition of prolamines and variability of economically beneficial traits, which is so important for the progress of plant breeding. The studies of this kind are being conducted at the All-Union Research Institute for Plant Breeding and Genetics. Some research results have already been published. This paper presents the results of the studies on the use of genetically determined polymorphism of gliadins in wheat breeding.

Material and methods

Old and new wheat variaties were used in this study as well as the lines developed from them (biotypes), and especially developed hybrids of various generations. The chromosomes carrying loci coding for gliadin of cultivars Odesskaya 3, Odesskaya 16 and Odesskaya 26 were identified based on the results of the examination of gliadin of the F_1 and F_2 monosomic and disomic hybrids from crossing Chinese Spring monosomics with the above mentioned varieties.

Proteins intended for electrophoresis were extracted from flour of 30-70 p. 100 yield by 70 p. 100 ethanol solution at room temperature. The protein from the second extract was used for separation.

Electrophoresis was carried out in starch gel in glass tubes. The tubes were 6 mm in diameter and 150 mm in length. Buffer was prepared from aluminium lactate (1.5 g per l) and lactic acid (pH up to 3.1). Urea was added to the gel to bring concentration to 3 M. About 300 mg of the protein in ethanol solution were placed into the tube. A dehydrated hydrolyzed starch was placed into the tube as an anticonvection medium. Power was 1 mA, and voltage 200-250 V. Electrophoresis lasted for 4-4.5 hours. To have protein fixed the gels were forced out into 5 p. 100 TCA solution by a rubber ballon type ball. For this purpose special racks were made at the Laboratory major part of which were glass tubes (bottomless) 10-11 mm in diameter. Fifteen-twenty minutes

later the gels were transferred to a 2 p. 100 nigrosin solution. Staining lasted for 30-45 minutes. Extra dye was washed off the gels with heated (up to 20-25 °C) running tap water.

Results

When gliadin composition of the F_1 seeds of the different crosses was examined it was established that all the components unique to both parents were present in the heterozygous endosperm. However, new hybrid proteins are not formed. In the F_1 seeds of reciprocal crosses, the components of the maternal variety are more distinctly expressed. All this indicates that the gliadins are inherited according to the codominant type and protein synthesis proceeds in the heterozygous endosperm in accordance with the maternal and paternal gene dosage ratio (6, 8).

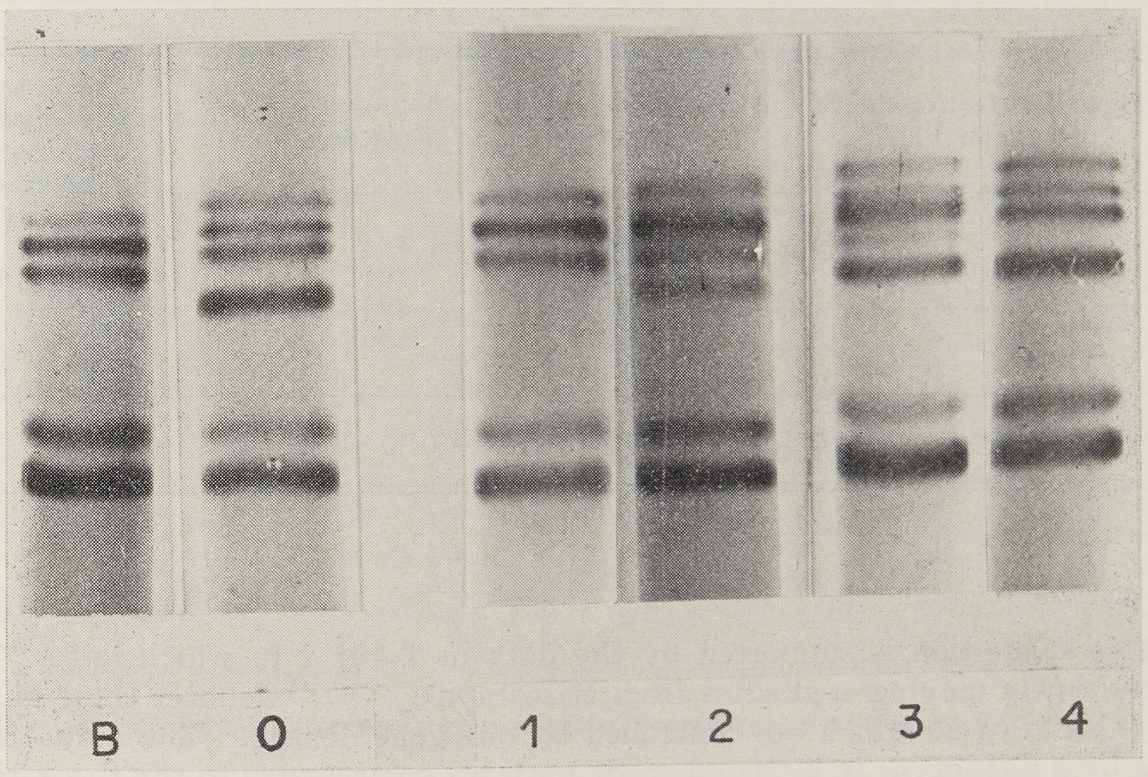

FIG. 1. — *Electrophoretic patterns of ω-gliadins of Bezostaya 1 (B), Odesskaya 26 (O) and all encountered types of electrophoretic patterns of F_2 single seeds from their crossing.*

In our early work on the inheritance of gliadins analysis of single F_2 seeds of the Bezostaya I × Odesskaya 3 cross revealed that components 9 and 10 of Odesskaya 3 gliadin were inherited in a linkage form (6). This fact, however, has been long ignored. Similar evidence from other crosses enabled us to shape a concept of a block of components (9, 3, 10). These allelic blocks of gliadin components were first identified in the analysis of the single F_2 seeds of Bezostaya 1 × Odesskaya 26 crosses (10). Figure 1 shows electrophoretic profiles of ω-gliadins

from Bezostaya 1, Odesskaya 26 wheats and all encountered types of profils of single F_2 seeds of the crosses between these varieties. Less mobile ω-gliadins from Bezostaya 1 are represented by three components and those of Odesskaya 26 by four components. The F_2 seeds in this zone of the profile gave four types of electrophoretic patterns. The first type does not differ from the pattern of Bezostaya 1 and the fourth — from the pattern of Odesskaya 26. The second and the third types may be regarded as heterozygotes. In the second type, however, the components unique to Bezostaya 1 are manifested more distinctly whereas in the third one more distinguishable are those from Odesskaya 26. No other combinations of components in this zone were found, neither electrophoretic patterns with missing components. These findings suggest that the first three gliadin components from Bezostaya 1 and the first four components from Odesskaya 26 were inherited in a linkage form and that they were controlled by allelic loci.

TABLE 1

Segregation for slowly moving ω-gliadins based on the analysis of single F^2 seeds of Bezostaya 1 × Odesskaya 26 cross

Type of a electrophoretic pattern in Fig. 1	Endosperm genotype	Number of seeds	χ^2	P
1	BBB	103		
2	BBO	102	0.10	> 0.99
3	OOB	105		
4	OOO	106		

Note χ^2 at the theoretically expected segregation, 1:1:1:1.

This suggestion is supported by the data in Table 1 in which letter " B " denotes slowly moving ω-gliadins from Bezostaya 1 " O " denotes these gliadins from Odesskaya 26 which are controlled by one gene dosage. The actual segregation by a phenotype in electrophoretic patterns corresponds to the theoretically expected segregation by genotype of endosperm; 1 : 1 : 1 : 1. It gives the evidence that the groups of components concerned are controlled by allelic loci which are apparently composite (polygenic).

Thus, the block is a group of components in the electrophoretic profile which are inherited in a linkage form, mendelian and non-recombinant in the process of crossingover. Allelic blocks may differ in the number of components. Under this aspect it is easy to explain the nature of varietal differences in gliadin electrophoretic patterns with similar number of loci coding for gliadin in these varieties.

Monosomic analysis demonstrated that the first four gliadin components from Odesskaya 26 are controlled by chromosome 1D (II). By using the allelism criterion it is possible to conclude that the first three gliadin components from

Bezostaya 1 are also controlled by the 1D chromosome. These varieties may be used at present to identify new types of blocks controlled by the 1D chromosome.

The results of identification of blocks of components in common hybrids and data from the monosomic analysis enabled us to use a genetic principle for gliadin classification, the basis of which is a block of components. According to the proposed nomenclature (5), the block of components from Odesskaya 26 controlled by the chromosome 1D is designated as Gld 1D4 and its allelic block from Bezostaya 1-Gld 1D1. The symbol Gld is an abbreviated name of protein (gliadin), following it a figure and a letter indicate chromosome number and genome responsible for the synthesis of the block of components, and the figure following the letter — ordinate number of the block of components controlled by this chromosome and registered in the catalogue of blocks.

TABLE 2

Segregation for gliadin component blocks controlled by the 1A and 1B chromosomes in the analysis of F_2 plants from the Bezostaya 1 × Triumph cross

Type of an electrophoretic pattern of gliadin in Fig. 3	Plant genotype according to blocks of components controlled by chromosomes		Number of plants in classes at $n = 118$		χ^2	P
	1A	1B	theoretically expected	actual		
1	4.4	1.1	7.11	8		
2	4.7	1.1	14.22	12		
3	7.7	1.1	7.11	5		
4	4.4	1.8	14.22	11		
5	4.7	1.8	28.44	34	4.64	> 0.75
6	7.7	1.8	14.22	17		
7	4.4	8.8	7.11	7		
8	4.7	8.8	14.22	14		
9	7.7	8.8	7.11	11		

Note: χ^2 calculated at the theoretically expected segregation, 1:2:1:2:4:2:1:2:1.

The blocks of gliadin components may also be identified in the protein extracted from the seed mixture from single F_2 plants. However, in this case, with codominant inheritance of a pair of allelic blocks (tentatively, A and B), segregation by a genotype proceeds rather in the ratio 1 : 2 : 1 than 1 : 1 : 1 : 1. It is brought about by the fact that the two classes of heterozygous F_2 plants in contrast to heterozygous seeds, are not distinguishable. Each of these plants has seeds of the endosperm genotype, AAA, AAB, BBA and BBB in the ratio I : I : I : I. It results in the dosage of allelic genes and their products in all heterozygous plants to be practically similar.

This method was applied to identify the blocks of components in one of the biotypes of Triumph (fig. 2 and 3). Previous experiments showed that in Bezostaya I, the block of components Gld 1A4 includes components 11 and partly 9 of the pattern and the block Gld IBI — components 4, 5, 6, 7, 8, partly 9, 17 and 18 (see Fig. 2). Figure 3 presents all pattern types encountered in F_2 plants of the Bezostaya I × Triumph cross. The first type is identical to the gliadin pattern from Bezostaya 1. In the third type block Gld 1A4 is missing. However, it has a considerably increased intensity of the component similar in electrophoretic mobility to the fourth gliadin component from Bezostaya 1, and it possess components 4,5 and 12 unique to Triumph. In addition, the plants with the third

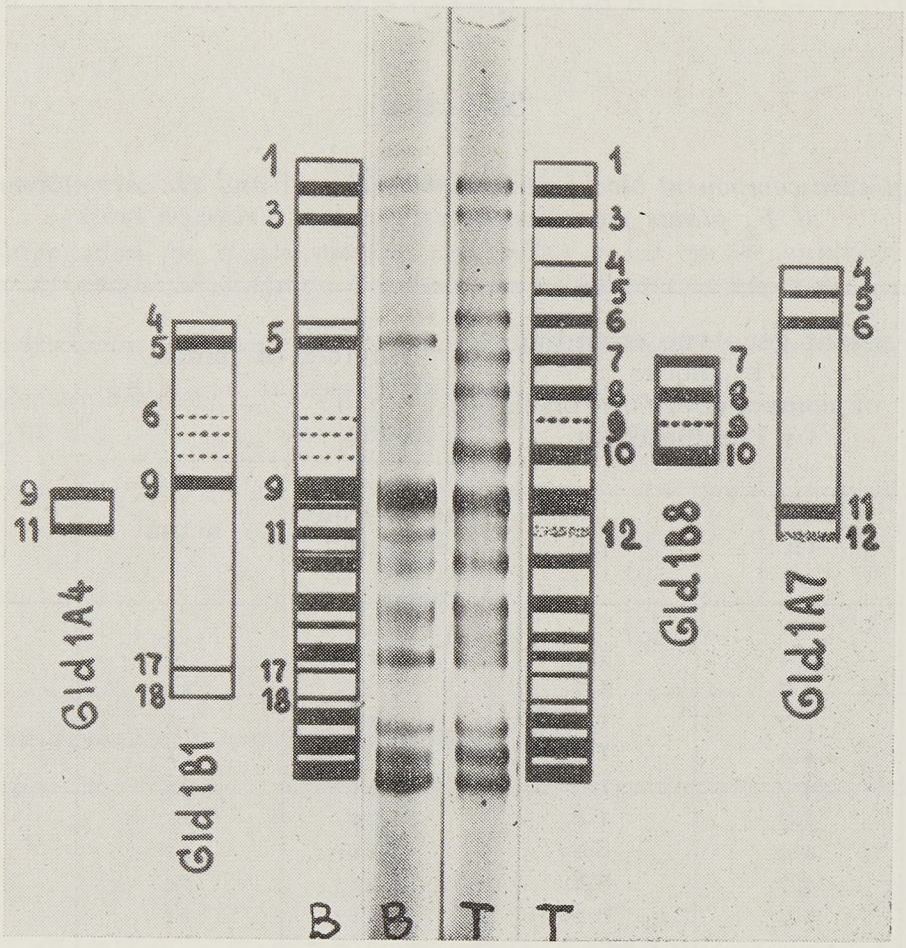

FIG. 2. — *Electrophoretic patterns and schemes of electrophoretic patterns of Bezostaya 1 (B), Triumph (T) gliadin and blocks of components controlled by chromosomes 1A and 1B.*

type of the pattern have a markedly increased intensity of the component similar in electrophoretic mobility to component 9 of gliadin from Bezostaya 1. I suggests that components 4, 5, 6, 12 and partly component II from the Triumph pattern make up a block allelic with block Gld 1A4. It is designated as Gld 1A7. The plants with the second type of the pattern may be regarded as heterozygotes for these blocks. There is no block Gld IB I in the plants with 7-9 types of profiles. In all these plants this block is replaced by components 7, 8 and 9 from Triumph. This group of components is designated as block Gld IB8.

With the plants having 7-9 types of patterns it is very convenient to observe the differences in blocks of components controlled by the IA chromosome. Most

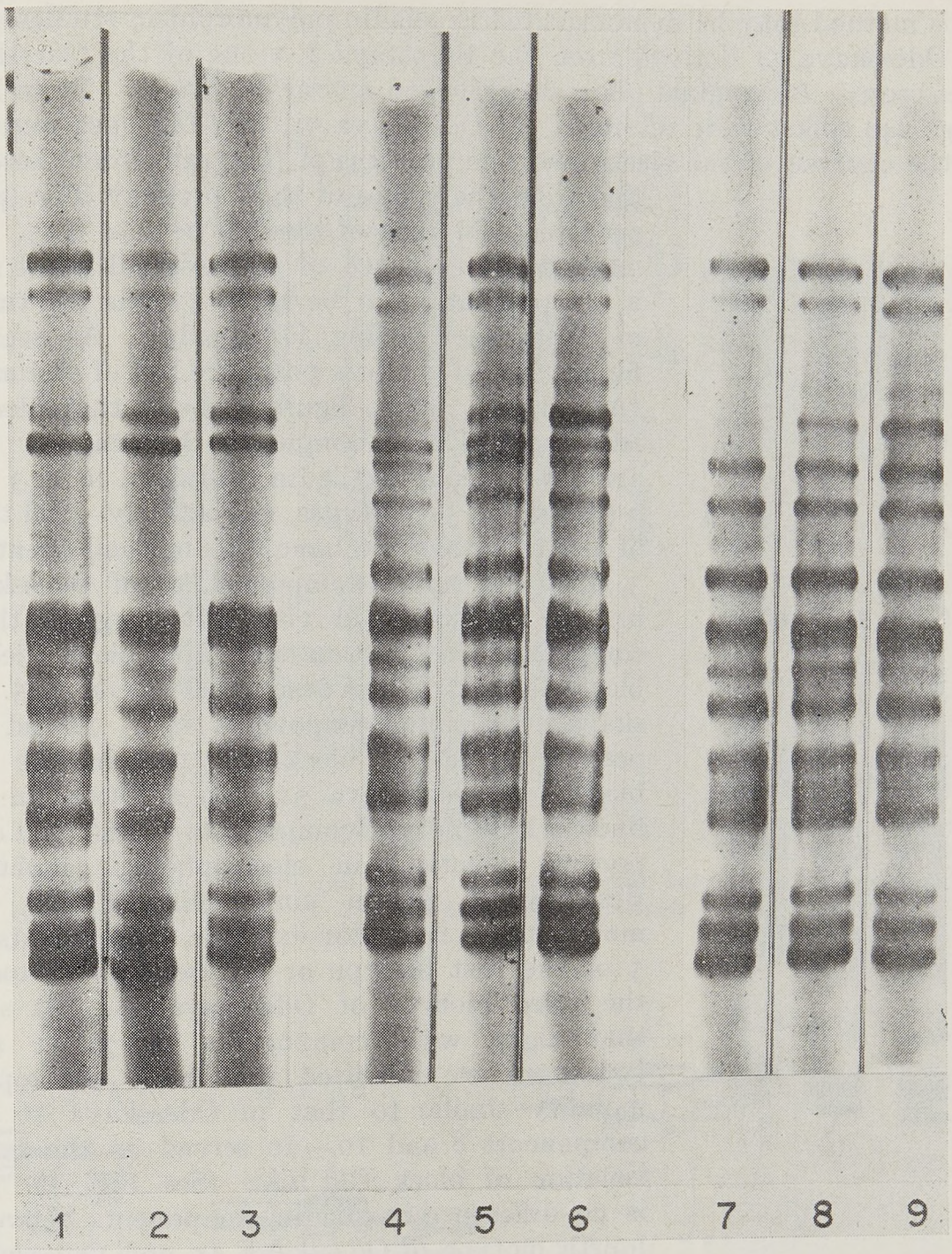

FIG. 3. — *Electrophoretic patterns (1-9) of gliadin in F_2 plants from the Bezostaya 1 × Triumph cross.*

of the components of the Gld 1A7 block from these plants are not masked by the components from other blocks. All the plants with 4-6 types of patterns are heterozygous for blocks Gld 1B1 and Gld 1B8. As for the blocks of components controlled by the IA chromosome the above regularity reoccurs. The actual segregation observed in the experiment corresponds to the theoretically expected one (Table 2), and this confirms the validity of the extraction procedure for gliadin blocks in Triumph.

As an alternative method of identification of blocks of gliadin components we also widely use the analysis of constant hybrids resulted from simple crossings. Here we may evaluate minor components in the patterns more accurately. It is very difficult to study the inheritance of these components in early segregating hybrid populations in the presence of heterozygotes.

This method may be demonstrated in gliadin polymorphism studies of widespread Odesskaya 51 derived from the Bezostaya 1 *x* one of the Odesskaya 16 biotypes cross. Its original plant was selected among F_2 plants. From F_{15} elite plantings 240 spikes were selected. In Odesskaya 51, four biotypes were identified in the analysis of the electrophoretic patterns of the grain from these spikes and from the spikes of their progeny (Fig. 4). The occurrence of each of them is near to 0.25. This is an indirect evidence of the original plant selected in F_2 population to be heterozygous for two pairs of allelic loci coding for gliadin. As seen from figures 4 and 5 these pairs are loci of chromosomes IA and ID. Thus, figure 4 shows that blocks Gld IA4 and Gld 1D1 unique to Bezostaya I (Fig. 5) are constituents of gliadin contained in 1, 3 and 1, 2 Odesskaya 51 biotypes, respectively. All the lines of the third biotype have gliadin components 1, 2, 3, 4, 5 and minor component 10 of Odesskaya 16 instead of block Gld 1D1. It suggests that the components in question make up a block allelic with block Gld 1D1. It is designated as Gld 1D5. Odesskaya 16 gliadin components 6, 7, 11 and 16 are present instead of block Gld 1A4 in the second biotype of Odesskaya 51. In addition, in all the lines of this biotype components 6 and 7, i.e. components identical in electrophoretic mobility to Bezostaya 1 gliadin components 4 and 5, are more intense than, for example, components 4 and 5 of the first biotype or components 6 and 7 of the third biotype of Odesskaya 51. It suggests that along with components 6, 7, 11 and 16 two more are inherited that have electrophoretic mobility similar to that of Odesskaya 16 gliadin components 8 and 10. It served as the basis for isolation of block Gld 1A2 (See Fig. 5). There is no difference in gliadin components between the fourth biotype of Odesskaya 51 and Odesskaya 16. Thus, analysis of constant lines made it possible to find out in what blocks of the Odesskaya 16 gliadin, minor components 10 and 11 were contained. Analysis of F_2 plants did not help to clarify this matter.

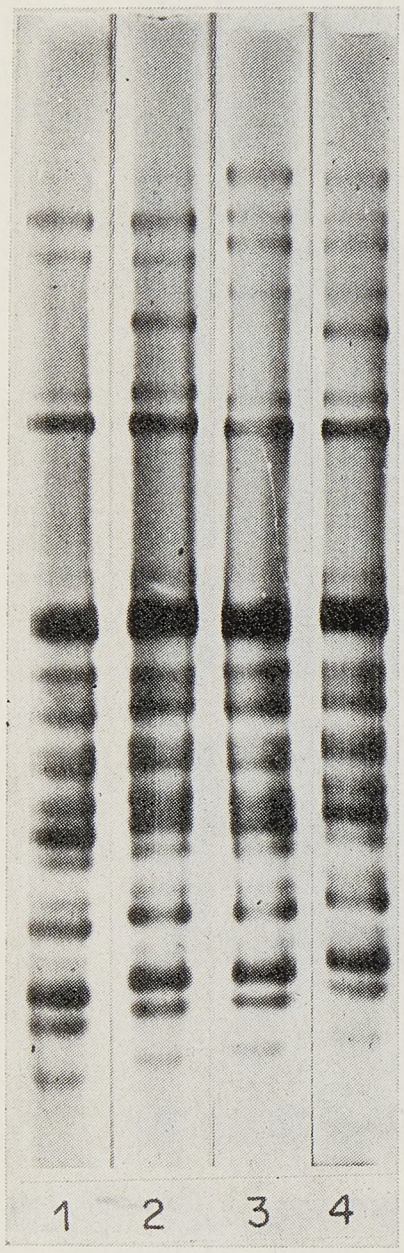

FIG. 4. — *Electrophoretic patterns of gliadin in Odesskaya 51 biotypes (1-4).*

Allelic blocks of gliadin components controlled by chromosomes 6A, 6B and 6D were identified similarly. Mironovskaya 808 gliadins and most of identified by us blocks of gliadin components controlled by loci of wheat chromosomes of the first and sixth homoeologous groups.

The resultant electrophoregram of gliadin of wheat variety, line or hybrid may be presented in the form of a set of gliadin component blocks. As Figure 7 shows Bezostaya 1 gliadin contains component blocks Gld 1A4, Gld 1B1, Gld 1D1, Gld 6A1, Gld 6B1, Gld 6D1. Gliadin of the first biotype of Odesskaya 16 has blocks Gld 1A1, Gld 1B1, Gld 1D5, Gld 6A3, Gld 6B2, Gld 6D1.

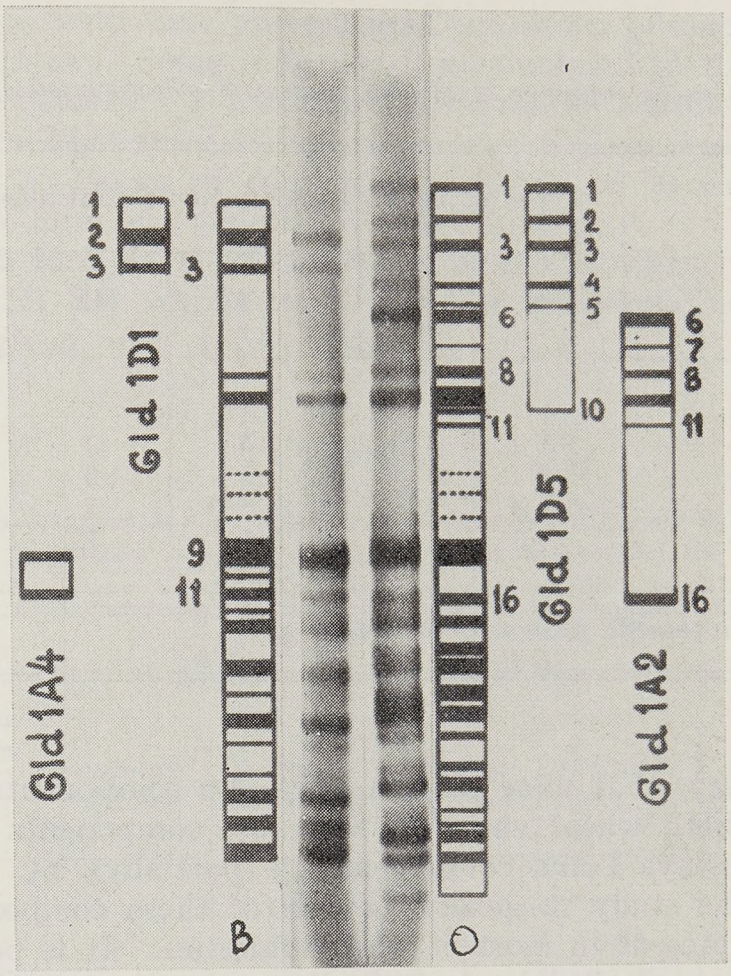

FIG. 5. — *Electrophoretic patterns and schemes of electrophoretic patterns of gliadin in Bezostaya 1 (B), Odesskaya 16, biotype 11 (O) and blocks of components controlled by chromosomes 1A and 1D.*

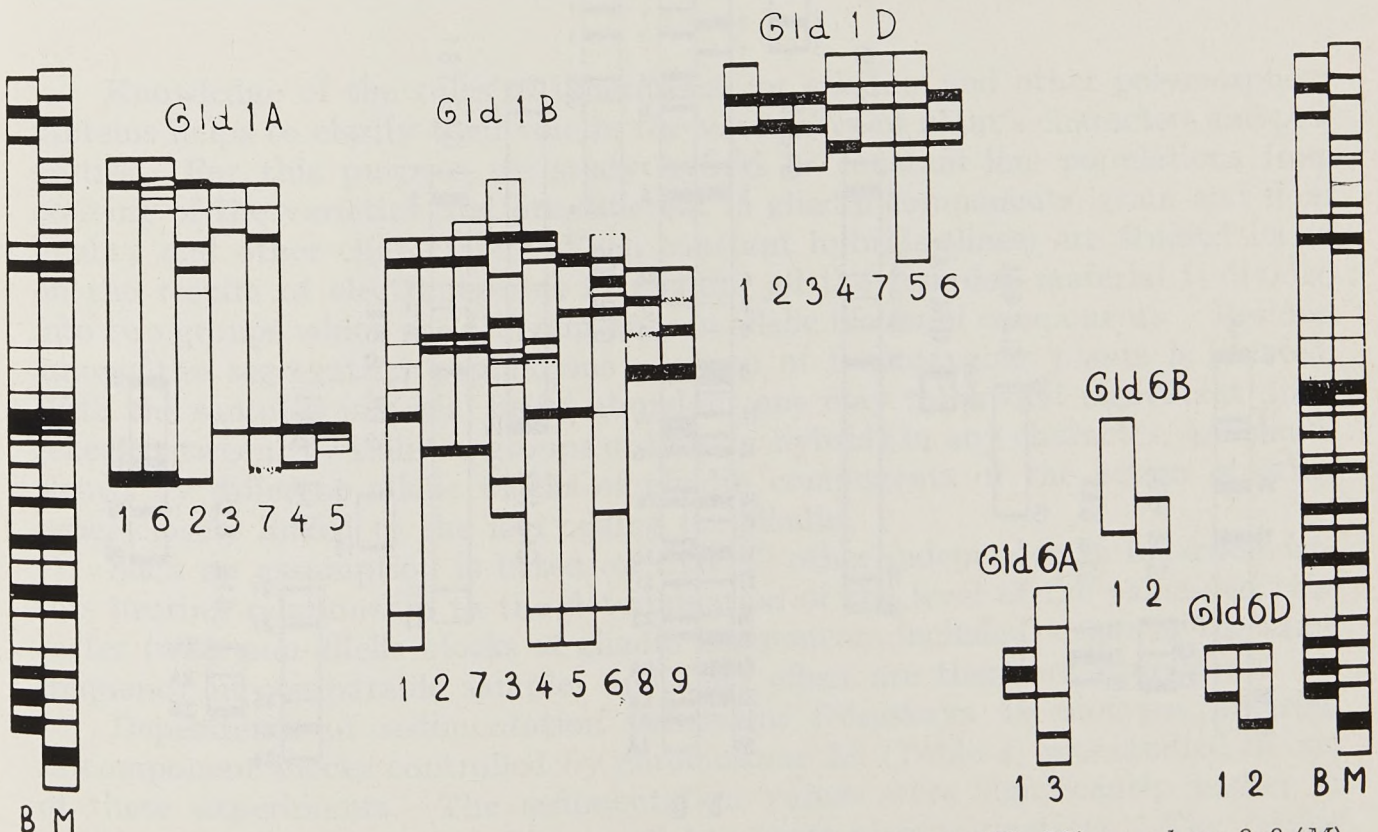

FIG. 6. — *Schemes of electrophoretic patterns of gliadin in Bezostaya 1 (B), Mironovskaya 808 (M) and identified heretofore blocks of gliadin components.*

TABLE 3

Genetic formulae of gliadin in several wheat varieties

Variety	Types of blocks of components controlled by chromosomes					
	1A	1B	1D	6A	6B	6D
Bezostaya 1	4	1	1	1	1	1
Mironovskaya 808	3	3	5	3	1	2
Odesskaya 16	1 + 2	1	5	3	2	1
Odesskaya 51	2 + 4	1	1 + 5	3	2	1
Dneprovskaya 521.	1	2	1	1	1	1

Fig. 6 show electrophoretic patterns of Bezostaya 1.

Figure 7 displays that some portion of gliadin components does not belong to any blocks in both wheat varieties: these are components 9 (partially), 12, 13, 14, 16 in Bezostaya I and components 13 (partially), 15, 16, 18 and 20 in Odesskaya 16. The study in genetic control of these components is impeded by that they are present in most of wheat varieties. It is possible to assume that they are also controlled by loci of chromosomes of homoeologous groups

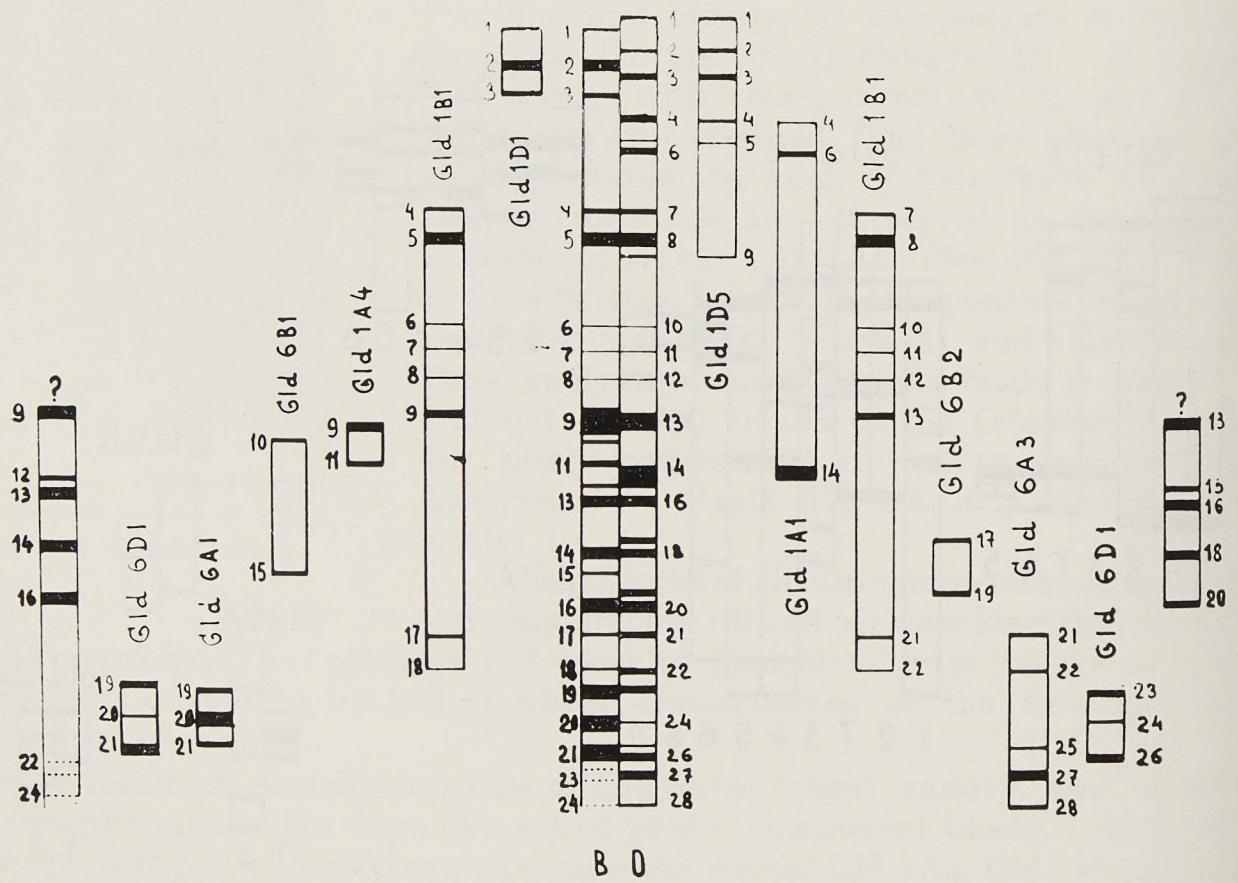

FIG. 7. — *Schemes of electrophoretic patterns of gliadin in Bezostaya 1 (B), Odesskaya 16, biotype 1 (O) and blocks of gliadin components unique to these cultivars.*

1 and 6. Therefore, with time proceeding, the number of components of blocks identified may increase. Some difficulty is encountered in the study of minor components of the pattern. For instance, it is true for Bezostaya I gliadin components 22-24 (See Fig. 7).

Identification of blocks of components enables us to express an electrophoretogram in the form of a genetic formula (Table 3).

In the gliadin formula there are types of blocks which are controlled by chromosomes IA, IB, ID, 6A, 6B and 6D. For heterogeneous varieties two or more blocks are given with a (+) symbol.

TABLE 4

Sedimentation values for Odesskaya 16 biotypes differing in blocks of gliadin components controlled by chromosome 1A

Biotype number	Gliadin formula	1976		1977		1978	
		number of lines	sedimentation, ml	number of lines	sedimentation, ml	number of lines	sedimentation, ml
1	1.1.5.3.2.1	33	63.5	53	45.0	53	35.4
2	2.1.5.3.2.1	46	70.9	81	52.4	80	41.7
	differences		7.4(*)		7.4(*)		6.3(*)

(*) Differences are significant at P = 0.001.

Knowledge of the rules of inheritance for gliadins and other polymorphous proteins helps to clarify their role in the variability of plant's characters and properties. For this purpose we study hybrid or constant line populations from crossing of the varieties that are different in gliadin components, grain and flour quality and other characters. When constant hybrids (lines) are studied based on the results of electrophoresis of gliadin, all the sampled material is divided into two groups which are different only in allelic blocks of components. Besides, among the segregating populations a group of heterozygous plants is isolated. With the sampled material being abundant one may think that significant differences between the isolated groups of lines or hybrids in any character, are conditioned by different allelic blocks of gliadin components or the action of other genes closely linked to the loci coding for gliadin.

Such an assumption is based on that all other independently inherited factors bearing relationship to the determination of the level of the examined character (with non-allelic blocks of gliadin components included) occur at the same frequency in comparable samples and their effect are therefore equivalent.

Dependence of sedimentation values for Odesskaya 16 biotypes differing in component blocks controlled by chromosome IA (Table 4) was studied in one of these experiments. The sedimentation values were significantly higher in the lines having block Gld IA2 in all the years of investigations. The results of this experiment were verified by comparing each of these blocks with block

Gld 1A4. For this purpose, the Odesskaya 51 lines differring in allelic blocks Gld 1A2 — Gld 1A4, Gld 1D1 — Gld 1D5 and specially made crosses between Bezostaya 1 and the first biotype of Odesskaya 16 having gliadin formula 1.1.5.3.2.1. were used.

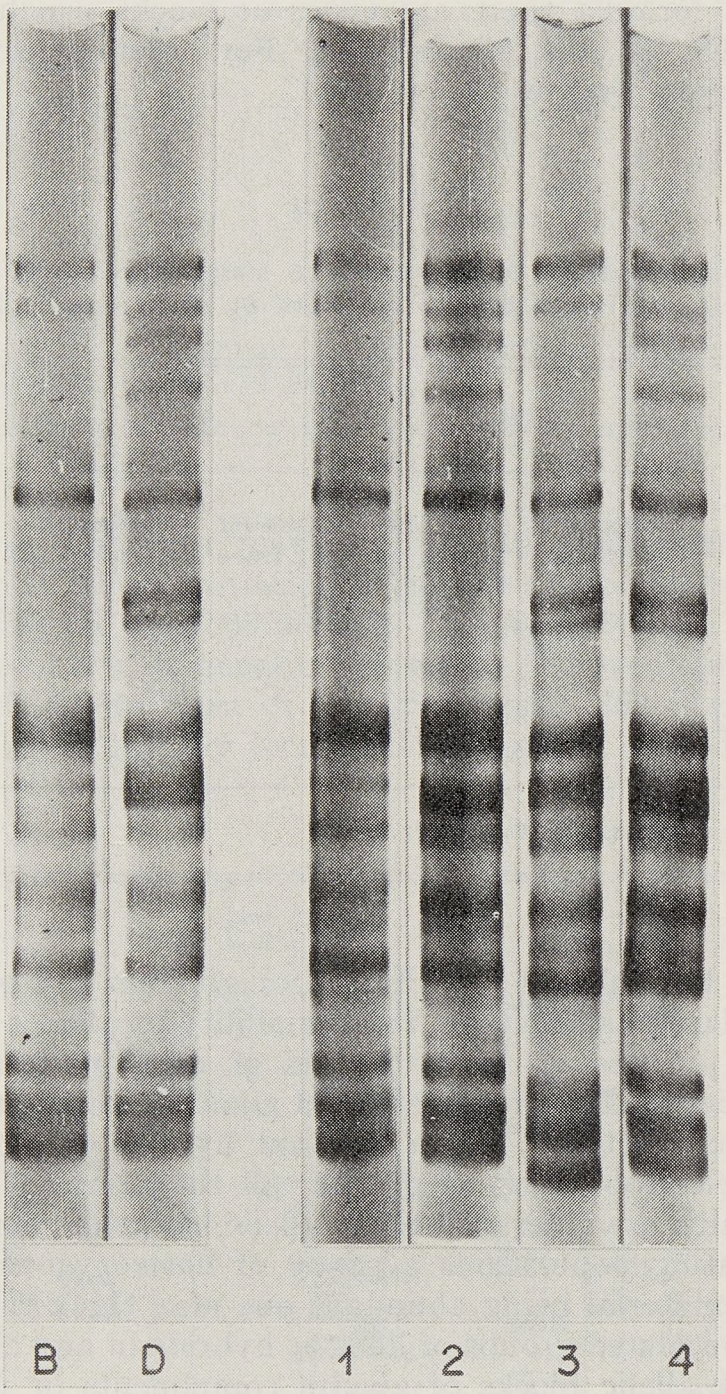

FIG. 8. — *Electrophoretic patterns of gliadin in Bezostaya 1 (B), Dneprovskaya 421 (D) and easily distinguishable types of electrophoretic patterns of the F_2 — F_4 plants from their crossing.*

It was found that in Odesskaya 51, blocks Gld 1A2 and Gld 1A4 exerted a minor effect on flour quality. The sedimentation value was 1.24 ml higher for biotypes having block Gld 1A4. However, in F_3 hybrids (Bezostaya 1 *x* the first biotype of Odesskaya 16) the sedimentation value on the average for the group of hybrids having block Gld 1A4, was 6.8 ml higher than the value for hybrids with block Gld 1A1 (at a high significance level).

The negative effect of block Gld 1A1 on flour quality is also confirmed in the study of the Bezostaya 1 *x* Dneprovskaya 521 crosses with gliadin formulae, 4.1.1.1.1.1. and 1.2.1.1.1.1, respectively.

In F_2 and subsequent generations of the hybrids derived from these varieties it is possible to isolate four plant types according to a phenotype in electrophoretic patterns (Fig. 8). The first and fourth types do not phenotypically differ from the parental varieties. In the second type of plants when compared to the electrophoretic pattern of Bezostaya 1 gliadin, block Gld 1A4 is replaced by block Gld 1A1. This class also includes the plants which are heterozygous for these blocks. In the plants having the third type of electrophoretic patterns when compared to Bezostaya 1, block Gld 1B2 takes the place of block Gld 1B1. It is also difficult to distinguish between heterozygotes and homozygotes according to these alleles. The plants of the fourth type of electrophoretic patterns can be homo-, or heterozygous for the examined pairs of allelic blocks.

The data in Table 5 enable us to suggest that the resultant segregation in the F_2 generation corresponds to the theoretically expected segregation in dihybrid crossing ($\chi^2 = 0.36$ at 1 : 3 : 3 : 9, P = 0.9).

TABLE 5

Mean sedimentation values in F_2 — F_4 hybrids of the Bezostaya I × Dneprovskaya 521 crosses varied with gliadin composition

Type of gliadin electrophoretic pattern in Fig. 8	F_2		F_3		F_4	
	number of plants	sedimentation, ml	number of plants	sedimentation, ml	number of plants	sedimentation, ml
1.	5	36.5	22	56.8	38	52,7
2.	17	29.5	38	48.3	34	42.5
3.	15	34.1	32	49.5	41	47.7
4.	42	29.3	72	45.8	49	39.5

TABLE 6

Effects of allelic pairs of blocks of gli adin components on sedimentation value in the F_1 — F_2 hybrids of the Bezostaya 1 × Dneprovskaya 521 crosses

Compared types of electrophoregrams in Fig. 8	Compared types of blocks	Differences if sedimentation values, ml		
		F_2	F_3	F_4
1-2.	1A4 ± to 1A1	+ 7.0(**)	+ 8.5(*)	+ 10.2(**)
3-4.	1A4 ± to 1A1	+ 4.8(**)	+ 3.7(*)	+ 8.2(**)
1-3.	1B1 ± to 1B2	+ 2.4(**)	+ 7.3(**)	+ 5.0(**)
2-4.	1B1 ± to 1B2	+ 0.2	+ 2.5	+ 3.0(*)

(*) Differences are significant at 0.05.
(**) Differences are significant at 0.001.

In all generations the hybrids of the first type of electrophoretic patterns have the highest sedimentation level while those of the fourth type have the lowest level. The data were statistically treated by comparing by pairs sedimentation values in plant groups differing only in allelic blocks (Table 6).

In all cases when plant groups differing in the blocks controlled by chromosome IA are compared, the sedimentation value is higher in the groups with gliadin containing block Gld IA4. The presence of block Gld IA1 unique to Dneprovskaya 521 is always associated with a significant reduction in the sedimentation value. Superiority of block Gld 1B1 over block Gld 1B2 is proved in the same way.

When sedimentation values for Bezostaya 1 and the hybrids with the first type of gliadin patterns are compared and also for Dneprovskaya 521 and the hybrids with the fourth type of patterns, differences in sedimentation values are small and not significant. It suggests that the differences in this character between parental varieties, Bezostaya 1 and Dneprovskaya 521, are conditioned by peculiarities of gliadin components. The major contribution to the differences is made by allelic blocks of chromosome IA.

In one of the experiments we studied the effect of blocks Gld IB1 and Gld IB3 on flour quality.

The Odesskaya 66 lines heterogenous for gliadin components were used in these studies (Odesskaya 66 was developed from the Kavkaz *x* Odesskaya 51 cross; Fig. 9. Table 7).

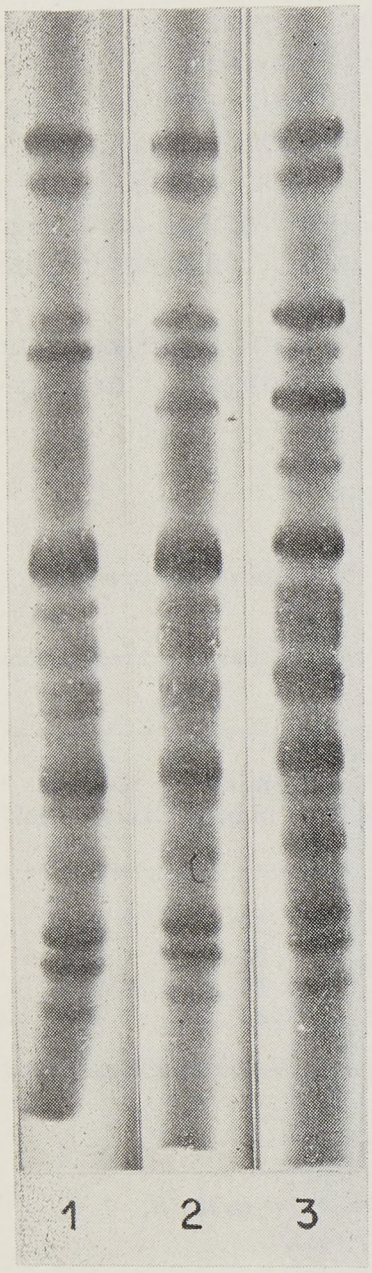

FIG. 9. — *Electrophoretic patterns of Odesskaya 66 biotypes*

1. — 4.1.1.3.2.1., 3. — 4.3.1.3.2.1.
2. — A mixture of the two biotypes.

In all the years the lines having block Gld 1B1 are of a better technological and baking quality. Presence of block Gld 1B3 in gliadin which is unique to all IB — IR translocation or substitution wheats is associated with deterioration of grain quality which in some years is rather severe.

Similarly, the role of the majority of identified blocks of gliadin components ω was clarified in the determination of the genetically conditioned flour quality (Table 8).

In some cases blocks of gliadin components can be used as reliable genetic markers of other economically important genes. For instance, block Gld IB3 is a reliable marker of gene(s) responsible for stem rust resistance in wheat (POPERELYA, BABAYANPZ, 1978). Three hundred and sixty eight F_8 — F_{10} lines from 25 crosses were studied in one of the experiments. In each cross one of the parents were either Aurora or Kavkaz which are distinguished for blook Gld IB3. Other varieties possessed block Gld IB1.

All of 163 lines with gliadin containing block Gdl IB3 turned to be resistant to stem rust. Out of the 205 lines with block Gld IB1, only 14 lines were resistant which is probably indicative of the action of other gene blocks.

TABLE 7

Grain and flour quality of the Odesskaya 66 biotypes differing in blocks Gld 1B1 and Gld 1B3

Characters	Year	Biotypes		Biotype 4.1.1.3.2.1 < to biotype 4.3.1.3.2.1
		4.1.1.3.2.1	4.3.1.3.2.1	
Sedimentation (ml)	1974	70	57	+ 13(**)
	1975	55	50	+ 5(*)
	1976	64	55	+ 9(**)
Grain:				
Protein	1975	16.10	16.40	— 0.3(*)
Content (%)	1976	13.40	13.40	—
Specific performance of dough deformation (e.u.)	1975	365	315	+ 50(**)
	1976	310	257	+ 47(**)
Bread volume (c.c.)	1975	858	840	+ 18
	1976	990	750	+ 240(***)
Bread porousness (scores) . . .	1975	4.63	4.38	+ 0.25(*)
	1976	4.20	2.50	+ 1.70(***)
Ratio h/d	1975	0.75	0.64	— 0.11(*)
	1976	0.55	0.37	— 0.18(*)

(*), (**), (***) Differences are significant at 0.05, 0.01 and 0.001 respectively.

The study of a large number of wheat samples from collections as well as hybrids and strains from wheat nurseries has allowed us to establish the relationship between gliadin composition and frost resistance in wheat (Table 9). The following blocks are found in gliadin of the highly resistant varieties: Gld IA1, or Gld IA2, Gld ID5, Gld 6A3, GId 6D2, or, probably, Gld IB2.

We did not find any variety of an intensive type with a high frost resistance (higher than in Bezostaya 1) missing the above blocks.

Our observations showed that all the winter wheats involving Bezostaya 1, were superior to it in this character only when the above blocks entered the composition of the gliadin.

Naturally, high frost resistance in spring varieties is not manifested with

TABLE 8

Relative value of allelic blocks of gliadin components in determination of flour quality

Gld 1A7 > Gld 1A4 > Gld 1A2 > Gld 1A5 > Gld 1A3 > Gld 1A1 ⩾ Gld 1A6
Gld 1B1 > Gld 1B2 ⩾ Gld 1B7 > Gld 1B5 > Gld 1B4 > Gld 1B3 > Gld 1B6
Gld 1D4 > Gld 1D5 > Gld 1D1 > Gld 1D2 > Gld 1D3
Gld 6A3 > Gld 6A1
Gld 6B2 > Gld 6B1
Gld 6D2 > Gld 6D1 ± Gld 6D3

any gliadin components. However, they can be successful donors of high frost resistance. For instance, as the results of our study show, Red River 68 possessing gliadin blocks Gld 6D2, Gld 6A3 and Gld 1B2 is one of such varieties. When crossed with Bezostaya 1, there were found the transgressive forms for frost resistance which possess these blocks.

TABLE 9

Gliadin components and frost resistance in wheat

Variety	Frost resistance	Gliadin formula according to blocks controlled by chromosomes					
		1A	1B	1D	6A	6B	6D
Alabasskaya	extremely high	1	1	5	3	1	2
Uljanovka	—	2	1	5	3	1	2
Odesskaya 16.	high	1 + 2	1	5	3	2	1
Kharkovskaya 159 . . .	—	1	1	5	3	1	1
Mironovskaya 808. . . .	—	3	1	5	3	1	2
Mironovskaya Jubileynaya	—	4	1	5	3	1	2
Mironovskaya 10	—	3	3	5	3	1	2
Byelotserkovskaya 198. .	—	5	1	5	3	1	2
Odesskaya 3	—	1	2	4	3	1	2 + 3
Krasnodarskaya 39 . . .	—	1	1 + 2	1	3	1	2
Dneprovskaya 521 . . .	above medium	1	2	1	1	1	1
Bezostaya 1	medium	4	1	1	1	1	1
Kavkaz	medium	4	3	2	1	1	1

Discussion

Our findings has allowed us to advance a genetic classification of storage proteins which is completely different from the existing biochemical classifications. Its basis are stable blocks of prolamine components which are inherited monofactorially and presumably are coded in wheat by polygenic loci (one in each of the homoeologous chromosomes of groups 1 and 6) and represented by multiple alleles. Since a block of components is taken as a classification unit it is not so important whether each component of the electrophoretic pattern is represented by one polypeptide, or by several proteins similar in electric charge. Actually, the block is a phenotypical marker of a polygenic locus at the level of protein molecules. The genetic classification of proteins is not an alternative to the biochemical one, but it enables one to obtain the information on the characteristics of a genotype and, therefore, it can be used to solve many problems of plant breeding and seed production.

The relationships between the allelic blocks and variation in traits such as technological qualities of the grain, low temperature tolerance and disease resistance, spike colour and etc., is not quite clear yet. However, the accumulating data suggest that prolamine allelic blocks are markers of gene blocks or nonrecombinant parts of chromosomes that play an important role in the formation of some characters.

It is quite possible that such relationships may be found in other crops. Thus, in the study of hordein polymorphism in barley, a correlation between allelic blocks of hordein and variability of some characters has been found. Correlation between polymorphism of other proteins and characters variability can be anticipated. The relationship of esterase composition with paddy grain size has been established (17).

It should be noted that the division of hybrid populations into two parts which are different only in one allelic block, is the principle method giving significant results. With this method, when there are many genotypes representing certain hybrid combinations, it is possible to study correlation between genetically determined polymorphism of other proteins, including enzymes, and variability of valuable traits. It is quite possible that every species possesses groups of proteins which are represented by multiple alleles, controlling variability in the manifestation of quantitative characters. These hypothetical " hot spots " of variability are of the most interest for plant breeding. The work in this field should be carried out by several stages — search for necessary polymorphic proteins, developments of their genetic classification, study of the relationship between their polymorphism and variation in characters.

At present the technique used to obtain electrophoretic patterns of prolamines and to develop their genotypical formulae, permits analysis of over one hundred genotypes per day. Regularities which have been established are widely used in wheat and barley breeding. Many scientists in various research institutions in the USSR and other countries have the opportunity to work at our Laboratory and to master the technique applied to identify blocks, record genotypical formulae of prolamines and define genotypes value for breeding purposes.

There is every reason to believe that the direction we are following in plant breeding, i.e. application of the achievements of biochemical and molecular genetics, will help to solve many practical problems of intensified plant breeding and primary seed production.

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Résumé

Les gliadines, extraites de grains F_1, F_2, F_3, F_4 de la descendance de différents croisements ont été examinées en électrophorèse verticale en tubes de gel d'amidon.

En F_1, il n'y a pas apparition de nouvelles protéines hybrides et la composition en gliadines indique que l'hérédité des composants est de type codominant. L'analyse des grains F_2 permet de définir le concept de descendance par « blocs » de composants associés et contrôlés par des lois allèles. L'analyse monosomique permet de déterminer la localisation chromosomique des gènes codant pour ces blocs de gliadines et de construire une nomenclature des blocs.

Une telle classification génétique des protéines de réserve est totalement différente de la classification biochimique existante. Les blocs constituent des marqueurs phénotypiques de loci polygéniques au niveau moléculaire, de sorte que cette classification génésique permet d'obtenir des informations sur les caractéristiques des génotypes.

Les données accumulées suggèrent également que les blocs allèliques prolamines sont des marqueurs de séries de gènes, ou de régions non recombinables de chromosomes, qui jouent un rôle important dans l'expression de caractères tels que les qualités technologiques du grain, la résistance au froid, la couleur de l'épi et la résistance aux maladies. Des résultats semblables devraient être découverts chez les autres céréales.

Bien que les relations entre ces blocs alléliques et les caractères considérés ne soient pas tout à fait claires, le fractionnement électrophorétique des gliadines est largement utilisé dans le domaine du blé et de l'orge et aidera à résoudre de nombreux problèmes de sélection végétale et de production de semences.

SESSION IV

Gluténines du blé

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Structure of glutenin : Achievements at the northern regional research center

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Summary

The Northern Regional Research Center (NRRC), Peoria, Illinois, is a laboratory of the U.S. Department of Agriculture's Science and Education Administration. For more than 20 years studies have been carried out on wheat gluten and its major fractions, gliadin and glutenin. This review summarizes studies of glutenin at NRRC and integrates this information with other laboratories' results to show how glutenin structure relates to wheat's functional properties.

In early studies at NRRC, glutenin was isolated and shown to be a heterogeneous mixture of high molecular weight (MW) molecules containing ordered and unordered structures. Molecules are asymmetric, and have high surface areas favoring interactions and association. Electrophoresis showed that reduced glutenin contains many subunits joined by intermolecular disulfide bonds; hydrogen and hydrophobic bonds also contribute to glutenin's structure. Three distinct types of glutenin subunits were isolated and characterized, and genetic control of glutenin's unique high MW subunits was established. Wheats having different qualities differ in subunit composition. Glutenin differs from low-MW gliadins, but its ethanol-soluble subunits are identical to subunits of high MW gliadin; a hypothesis was developed showing how different subunit types assemble into glutenin.

High MW native glutenin molecules are related to wheat quality. These molecules are oriented by mixing, and form disulfide and noncovalent bonds with other flour proteins, resulting in a continuous gluten network in dough. Gluten is cohesive because of the many covalent and non- covalent bonds between subunits; it is elastic because of changing interactions and conformations under stress, combined with a tendency to return to a minimun energy state.

Key words : *wheat — glutenin — structure — breadmaking — quality.*

(1) The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

Introduction

It is now more than 160 years since wheat gluten was first separated into two distinct fractions on the basis of differential solubility in alcohol, and more than 70 years since Osborne's classical studies, after which the term " glutenin " became accepted for its alcohol-insoluble fraction. Glutenin is widely recognized as an important contributor to wheat quality and functional properties but, in spite of numerous studies and perhaps due to its innate complexity, considerable divergence of opinion still exists as to the nature and even the existence of glutenin.

In part, this problem is due to glutenin's intractability: it is extremely difficult to solubilize, isolate, or purify without drastically changing its properties. As a result, many reports have considered only part of glutenin, and results and interpretations have differed. There is also no one good, simple definition for glutenin: it has meant different things to many people. For now, let us define glutenin as the complex, high-molecular-weight (MW) alcohol-insoluble fraction of wheat endosperm proteins made up of numerous subunits, joined both covalently and non-covalently, and including characteristic high MW subunits that are absent in all other wheat protein classes. This is not a simple definition, but it is broad and dynamic enough to allow incorporation of new knowledge.

The Northern Regional Research Center (NRRC) is located at Peoria, Illinois. It is part of the U.S. Department of Agriculture's Science and Education Administration (formerly the Agricultural Research Service). Research into the nature and structure of glutenin and other wheat proteins has been on-going at the laboratory for more than 20 years. During this time, as we have probed glutenin structure with new techniques, our understanding of its nature has improved. Our laboratory has published more than 50 research reports concerning glutenin composition, structure, properties, and quality. Other laboratories also have made significant and pioneering advances, of course, as detailed in recent reviews (WALL, 1979*b*; KHAN and BUSHUK, 1978; KASARDA *et al.*, 1976; BIETZ *et al.*, 1973; HUEBNER, 1977, 1978; HUEBNER *et al.*, 1977). This paper reviews NRRC research on glutenin and introduces new ideas and interpretations that permit a unified view of glutenin's structure. The many contributions of our colleagues at other laboratories, as detailed in the above-mentioned reviews, and their continued cooperation are gratefully acknowledged.

Initial studies

Studies at NRRC concerning the nature of wheat gluten and its components were first published in 1959; as new biochemical techniques became available during the next few years, additional studies yielded valuable new information about glutenin's structure. JONES *et al.* (1959) described methods for preparation of gluten and for its fractionation into gliadin and glutenin; these methods are still widely used today. JONES *et al.* also demonstrated, using moving boundary electrophoresis, that gliadin and glutenin are uniquely different (Fig. 1); glutenin had only one major peak, similar to the " α " peak of gluten, and was shown to

be associated with the gluten-like properties of wheat. The single peak observed for glutenin seems somewhat surprising considering current knowledge of ist heterogeneity and complexity; but, in fact, it may be an early indication that glutenin's subunits join randomly since their mass/charge ratio is relatively constant. Ultracentrifugal analysis (JONES *et al.*, 1961, 1964) found glutenin to be heterogeneous, having a weight average MW of 1.5-2 million, and to contain molecules with MW's ranging from about 50 000 to several million. MW's remained constant in several disaggregating solvents, suggesting that chemical

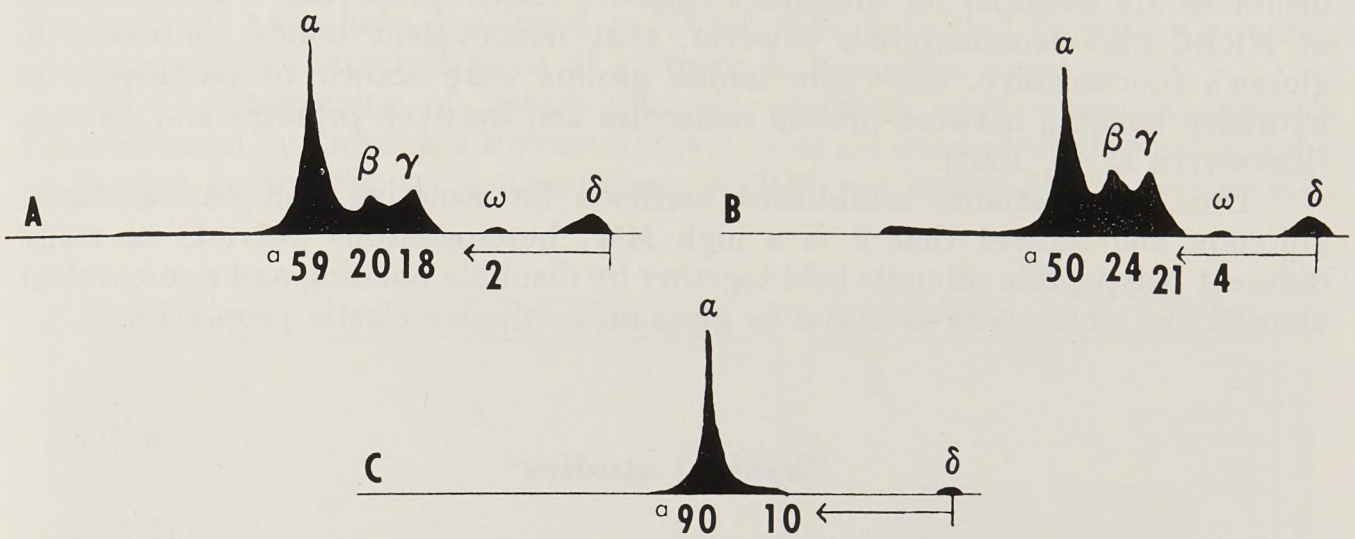

FIG. 1. — *Moving boundary electrophoresis of gliadin extracted from gluten ball with 70 p. 100 ethanol (A) and of gliadin (B) and glutenin (C) separated by neutralization of an acidic 70 p. 100 ethanol solution.* (From JONES *et al.*, 1959).

bonds, rather than physical aggregation, were responsible for glutenin's large molecular size. NIELSEN *et al.* (1962) confirmed native glutenin's MW distribution, but they also showed that disulfide bond cleavage reduced its MW to about 20 000. Reduced glutenin had lost its elastic and cohesive properties, showing that disulfide cross-links contribute to glutenin's native structure.

Good information on the amino acid composition of glutenin became available (WOYCHIK *et al.*, 1961; WU and DIMLER, 1963), and JONES *et al.* (1963) found that gluten could be fractionated into glutenin and gliadin by gel filtration on Sephadex G-75. The novel technique of starch gel electrophoresis (SGE) in aluminium lactate buffer (WOYCHIK *et al.*, 1961; JONES *et al.*, 1963) showed that native glutenin could not enter the starch gel. After reductive cleavage of glutenin's disulfide bonds, however, 20 or more components were released from glutenin (WOYCHIK *et al.*, 1964) (Fig. 2). Some were similar to

FIG. 2. — *Starch gel electrophoresis (SGE) of reduced gliadin (A) and glutenin (B).* (From WOYCHIK *et al.*, 1964).

gliadin, suggesting that glutenin was formed through disulfide bonding of gliadins; other components were obviously different. Thus, intermolecular disulfide bonding was proposed as a principal factor in glutenin's structure and unique rheological properties, which confirmed the studies of NIELSEN *et al.* (1961). It was proposed that gliadin modified glutenin's properties through disulfide interchange reactions. BECKWITH and WALL (1966) extended this concept by studying the reoxidation of reduced glutenin at various concentrations. Viscosity measurements during reduction suggested that both inter- and intra-molecular disulfides were present while reoxidation studies showed that appropriate ratios of these two types of disulfides are essential for glutenin's cohesive-elastic properties. Early studies at NRRC also demonstrated, however, that noncovalent bonds contribute to gluten's functionality: side-chain amide groups were shown to participate in hydrogen bonding between protein molecules and between proteins and solvents (BECKWITH *et al.*, 1963).

Thus, these studies established methods for isolating and characterizing glutenin, and showed that it is a high MW, heterogeneous polymer of many different polypeptide subunits held together by disulfide bonding and non-covalent associations which were essential to glutenin's cohesive-elastic properties.

Physical studies

Several studies at NRRC have characterized glutenin using the physicochemical techniques of intrinsic viscosity determinations, sedimentation velocity, titration. ultraviolet difference spectra, optical rotatory dispersion (ORD), osmotic pressure, circular dichroism (CD), and infrared absorption. TAYLOR and CLUSKEY (1962) found that glutenin behaves as a flexible, randomly coiled polyelectrolyte of high viscosity in solution, making it the major contributor to gluten's viscoelastic and rheological properties. WU and DIMLER (1963) estimated glutenin's ionizing groups by titration, which showed different conformations for gliadin and glutenin and changed conformation between acidic and basic pH ranges. WU and DIMLER (1964) used ORD, viscosity, and sedimentation velocity measurements to study glutenin in urea solutions at various pH's; glutenin was more asymmetric at pH 10 than at pH 4. Further studies established that glutenin contains both random coil and α-helical sequences (WU and CLUSKEY, 1965; CLUSKEY and WU, 1966). In glutenin, 10-20 p. 100 α-helix was found, which was somewhat less than in gliadin; helical content decreased in 3M urea. This relatively low α-helical content is consistent with glutenin's high proline content; the fact that α-helices exist at all probably reflects non-uniform proline distribution in gluten (CLUSKEY and WU, 1971). As the ionic strength of glutenin solutions increases, glutenin's helical content increases, and its intrinsic viscosity decrases significantly (WU *et al.*, 1967). These changes are not due to aggregation, since observed MW's do not change. This suggests that glutenin has a relatively high axial ratio and a somewhat loosely-held-together structure that is less constrained than that of gliadin. Glutenin's α-helix content can be varied from 0 to 35 p. 100 in 8M urea and trifluoroethanol, respectively, which disrupt and form α-helices. Thus, glutenin is an extended asymmetric molecule containing both ordered and unordered structures and having relatively high surface area; this structure promotes interactions with other proteins and flour constituents, giving rise to the rheological properties of gluten and dough.

Isolation and characterization of native glutenin

In most studies of glutenin, a compromise must be made between extractability and functionality. If mild extraction methods are used in the hope of maintaining functionality, incomplete glutenin recovery is achieved; if most or all glutenin is extracted, functionality characteristic of the native state is often lost. As a result, many studies have considered only soluble glutenin and not insoluble or " residue protein " fractions. A number of isolation and characterization methods have been developed at NRRC that, with that limitation in mind, have permitted characterization of glutenin in its native state.

The heterogeneity of native glutenin observed upon ultracentrifugation was confirmed by Crow and Rothfus (1968) using gel permeation chromatography on Bio-Gel P-300 (Fig. 3); a broad range of MW was indicated. However, SGE

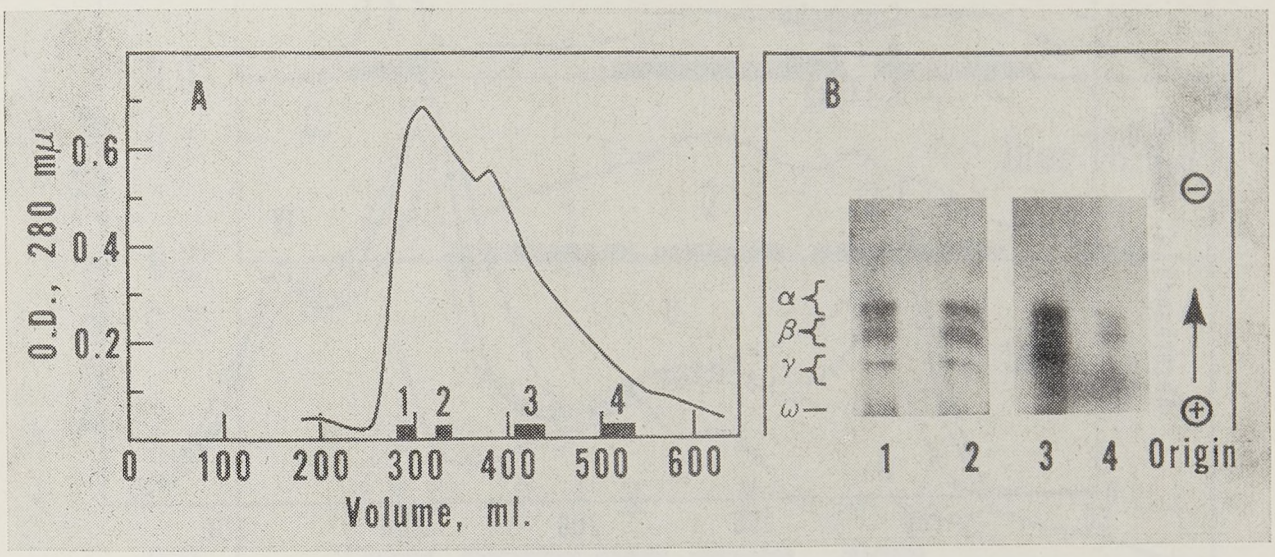

Fig. 3. — (A) *Gel filtration chromatography on Bio-Gel P-300 of native glutenin.* (*B*) *SGE of reduced and alkylated fractions from* (*A*). (From Crow and Rothfus, 1968).

of the reduced and alkylated fractions revealed similar subunit compositions, suggesting that glutenins of different MW's differ mainly in the degree to which subunits are repeated. Results also indicated that some proteins associate noncovalently with glutenin. Huebner (1970) also suggested, based on the shape of salt presipitation curves, that native glutenin consisted of several molecular species.

Huebner and Rothfus (1971) found that all glutenin was not extractable from flour by repeated extraction with 2M urea. When glutenin was subjected to gel filtration on Bio-Gel A-5m, an agarose gel with very large pores, two distinct peaks were obtained; after reduction and alkylation, they were shown by SGE to have significantly different subunit compositions. These results demonstrated that glutenin is highly aggregated, that it is difficult or impossible to extract completely, that it differs uniquely from gliadin, and that it is heterogeneous in its native state.

Bietz and Wall (1975) extended these studies by extracting native glutenin with a series of mild solvents. Glutenins extracted with dilute acetic acid and

with acetic acid-$HgCl_2$ were found to differ in subunit composition from each other and from residual glutenin extracted with α-mercaptoethanol, again confirming native glutenin's heterogeneity. Glutenins extracted under mild conditions were associated with much gliadin, demonstrating the absolute necessity of purifying extracted glutenins by reprecipitation. This study also established that " gel protein " and " residue protein " contain significant amounts of glutenin.

BIETZ *et al.* (1975) developed a method for studying glutenin's subunit composition in single wheat kernels. Albumins, and globulines, gliadin were first extracted, and then gliadin associated with glutenin was removed by first dissolving it in acidic 70 p. 100 ethanol and then reprecipitating all glutenin by neutralization. All glutenin remained in the residue and could then be solubilized (as subunits) by reduction with β-mercaptoethanol in the presence of SDS. Significant differences were demonstrated between glutenin extracted by this procedure and that prepared from a gluten ball; apparently certain subunits are only associated or

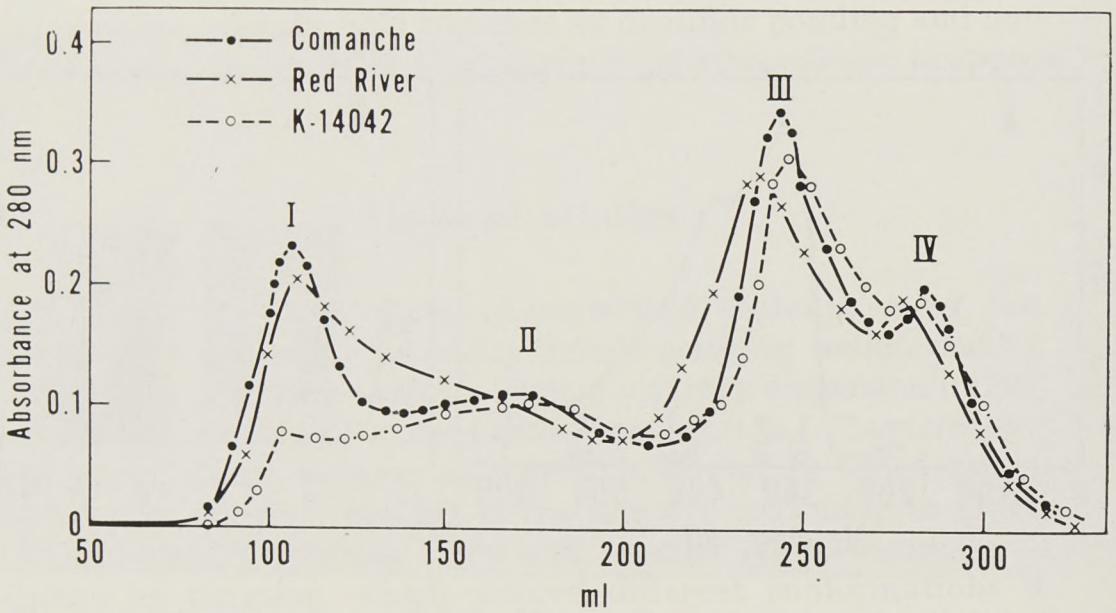

FIG. 4. — *Gel filtration of protein extracts from three wheat flours on Sepharose 4B.* (From HUEBNER and WALL, 1976).

loosely bound to glutenin and can be readily removed after dough formation. HUEBNER and WALL (1976) also demonstrated by gel filtration on porous agarose columns (Fig. 4) that native glutenin consisted of molecular species of at least two distinct sizes, having a wide range of apparent MW's of 20 000 000 or more (I) and from over 5 million to 100 000 (II). Amino acid analyses and SDS-PAGE of these fractions revealed little difference, suggesting that glutenin's disulfide cross-links are randomly formed *in situ*. Thus, these studies show that native glutenin is heterogeneous in MW and in subunit composition; these criteria are not simply related, however, and may also vary in different solvent systems.

Probably the best available picture of glutenin's true complexity is the recent study by HUEBNER and WALL (1980). Glutenin's solubility was found to vary from 25-50 p. 100 in 6M guanidine hydrochloride or 7M urea (at *ca.* 0.2 p. 100 concentration) to 75-85 p. 100 in SDS or sodium dodecanoate, demonstrating the importance of hydrophobic bonding in maintaining glutenin's structure. Gel filtration on agarose columns using the same solvents (Fig. 5) revealed complex

elution patterns for the soluble glutenins; MW's ranged from 37 000 to 10 million. High MW protein was present in all extracts, demonstrating intermolecular disulfide crosslinks; also present was low-MW protein (which was different from gliadin), previously associated with glutenin through hydrophobic or hydrogen

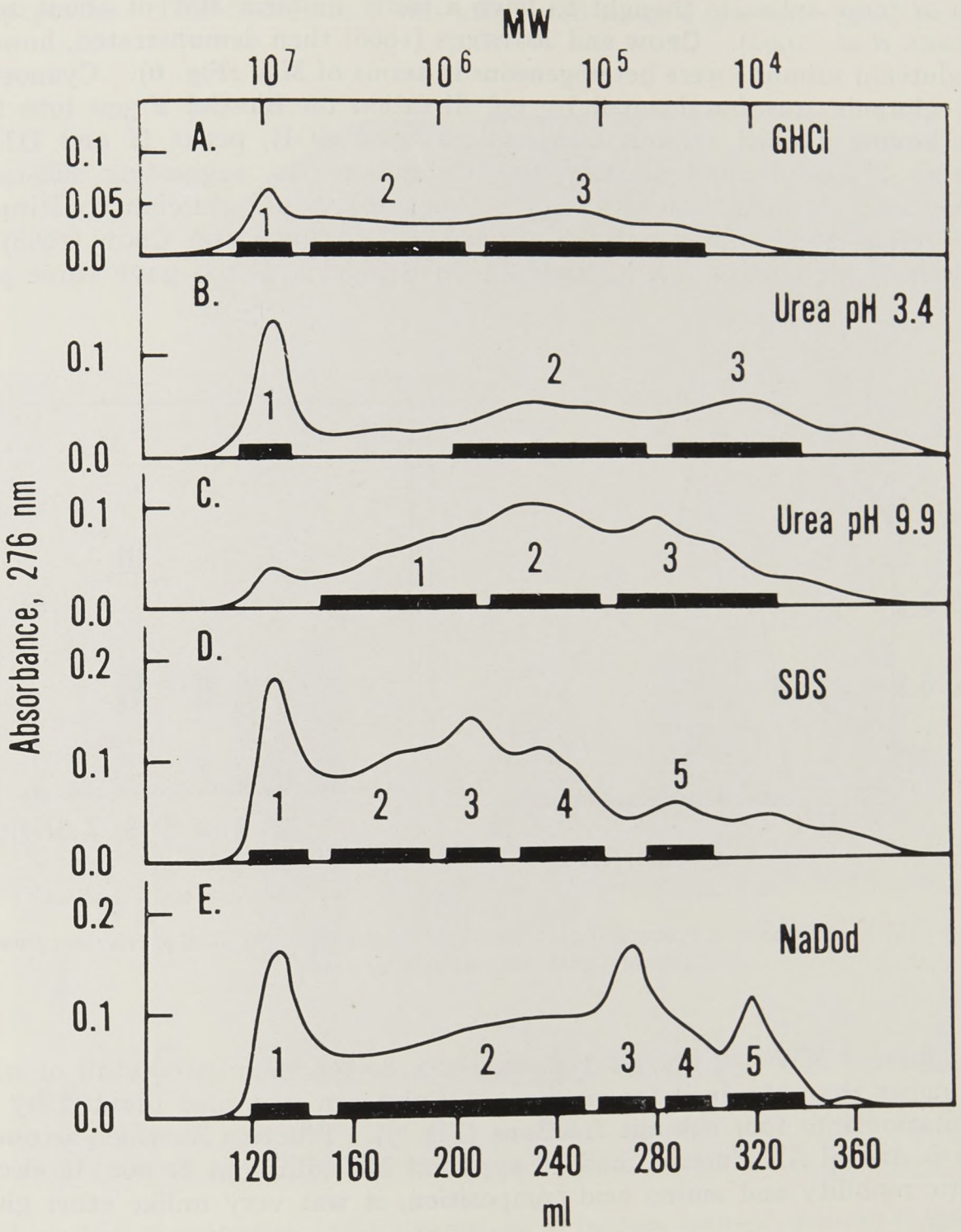

FIG. 5. — *Gel filtration on Sepharose CL-4B of glutenin in various dissociating solvents.* (From HUEBNER and WALL, 1976).

bonds. Fractions of glutenin with different MW's were shown by SDS-PAGE and amino acid analysis to differ in their subunit compositions, which suggests that the degree of disulfide crosslinking varies among fractions. Thus, glutenin has been shown to be heterogeneous in terms of MW, subunit composition, and types of bonding between subunits.

Isolation and characterization of glutenin subunits

As noted above, WOYCHIK *et al.* (1964) showed that reduced glutenin contained 20 or more subunits thought to have a fairly uniform MW of about 20 000 (NIELSEN *et al.*, 1962). CROW and ROTHFUS (1968) then demonstrated, however, that glutenin subunits were heterogeneous in terms of MW (Fig. 6). Cyanoethyl- (CN-) glutenin was fractionated by gel filtration on Bio-Gel P-300 into three peaks having distinct subunit compositions upon SGE; peaks II and III had apparent MW's of approximately 100 000 and 40 000, suggesting substantial compositional or structural variation between individual glutenin constituents. These results were subsequently confirmed by ROTHFUS and CROW (1968); gel filtration of aminoethyl- (AE-) glutenin on Sephadex G-100 gave three peaks

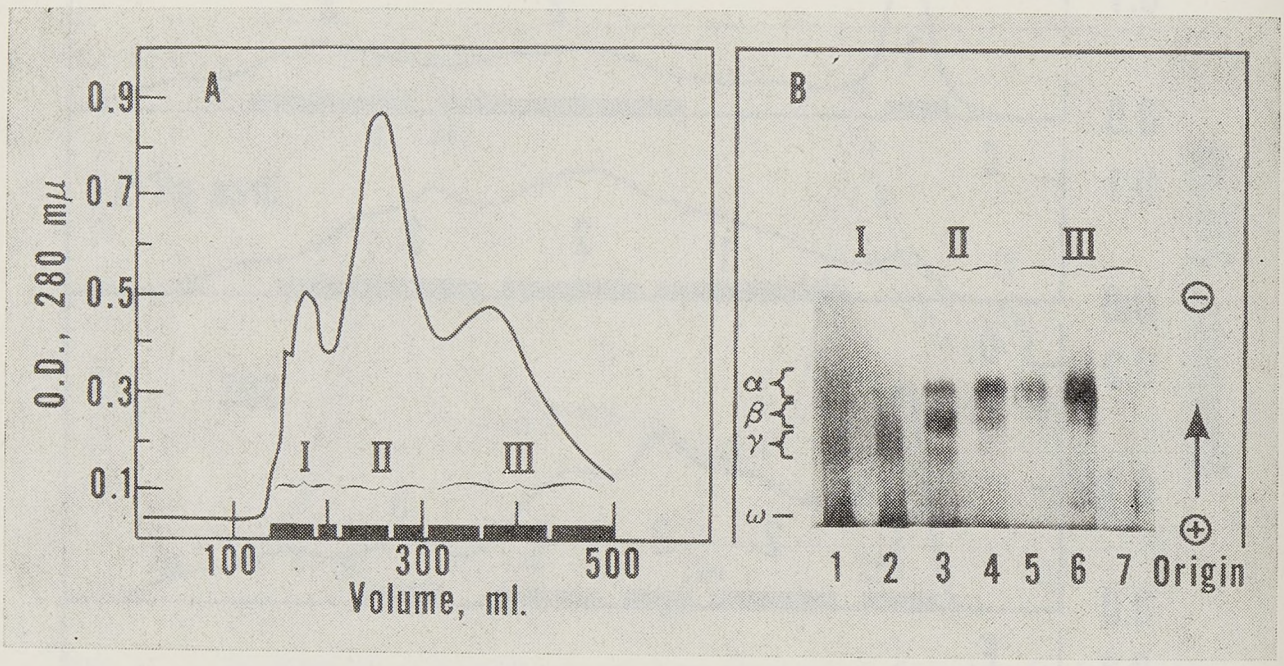

FIG. 6. — (A) *Fractionation of cyanoethyl-glutenin on Bio-Gel P-300.* (B) *SGE of fractions from* (A). (From CROW and ROTHFUS, 1968)

with apparent MW's of greater than 100 000, 80 000, and 40 000, all of which were higher than obtained for gliadin. AE-glutenin was also divided by salt precipitation into four subunit fractions (Fig. 7). Fraction A, which accounted for 22 p. 100 of AE-glutenin, had an apparent MW of about 80 000; in electrophoretic mobility and amino acid composition, it was very unlike other gluten proteins.

The next major advance in characterizing glutenin's came about through SDS-PAGE, which separates polypeptides solely on the basis of MW. BIETZ and WALL (1972) found that glutenin has subunits of at least 15 distinct apparent MW's (Fig. 8), ranging from 11 600 to 133 000. No remaining covalent crosslinks or association could be demonstrated, which suggests that these are the fundamental structural units of glutenin.

Recent studies, however, suggest that the MW's obtained for the slowest SDS-PAGE bands of glutenin may be too high. SEXSON *et al.* (1978) have con-

firmed a previous report (HAMAUZU *et al.*, 1975) indicating that glutenin subunit MW's estimated by SDS-PAGE were higher than obtained by sedimentation equilibrium. Using various dissociating solvent systems, SEXSON *et al.*, found apparent weight-average molecular weights of 55 000 to 66 000 for glutenin subunit fractions which, according to SDS-PAGE, are enriched in 87 000 to 133 000 MW polypeptides. This discrepancy was attributed to an apparently more elongated

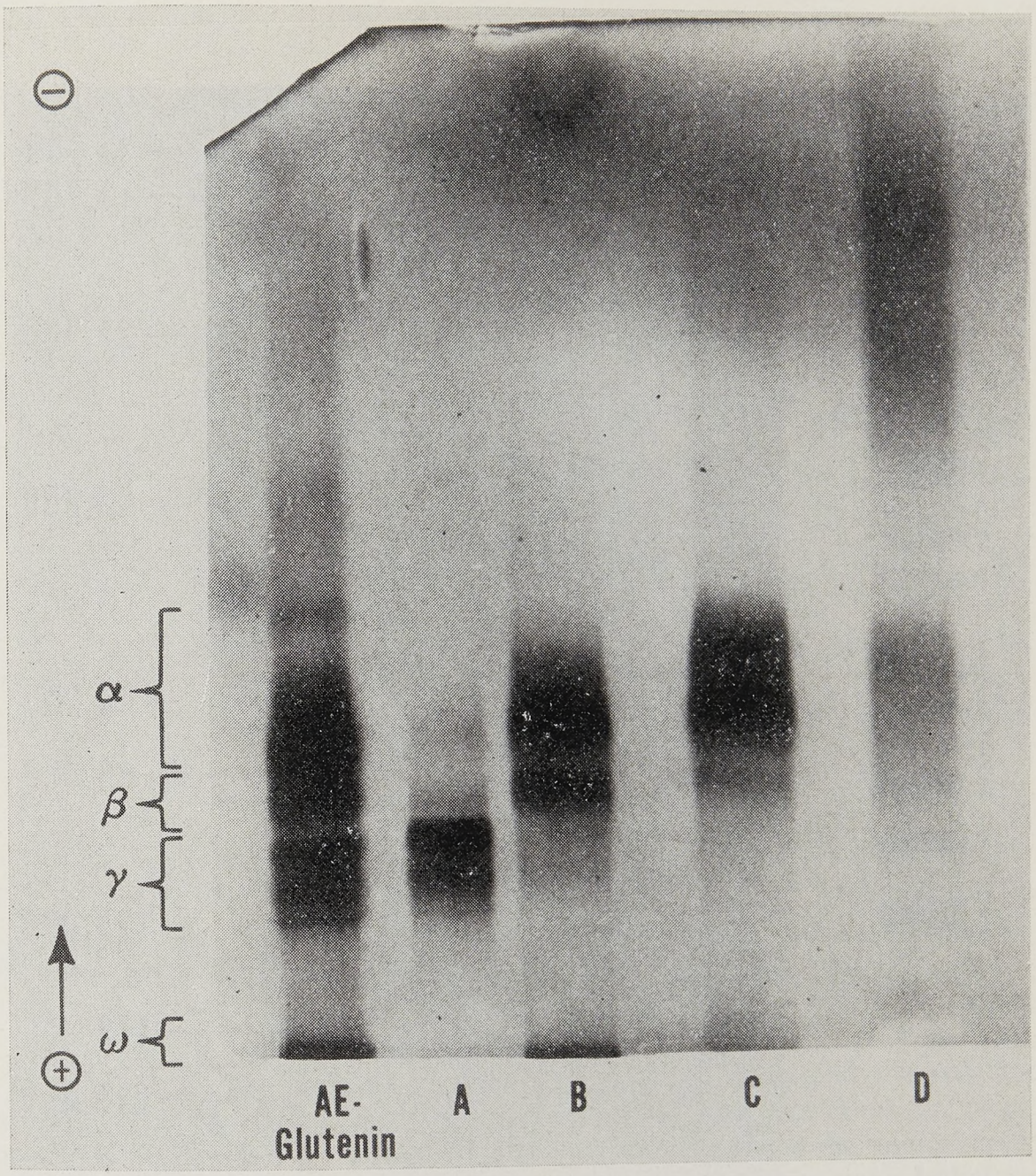

FIG. 7. — *SGE of aminoethyl- (AE-) glutenin and its fractions obtained upon salt precipitation.* (From ROTHFUS and CROW, 1968)

structure of glutenin polypeptide-SDS complexes than occurs for most proteins, due to the high content of proline in glutenin, which disrupts α-delices. Further studies are necessary to determine if the subunits really are more elongated, and perhaps only detailed structural analysis on individual low-mobility glutenin polypeptides will reveal their true MW's. Until such information is available, however, it is reasonable to accept the conclusion that apparent MW's of the

glutenin subunits having low SDS-PAGE mobilities are considerably overestimated.

Most glutenin subunits (Fig. 8) differed uniquely from gliadin, but subunits of apparent MW's 36 000 and 44 000 were observed in both fractions. These glutenin subunits were found to be similar to gliadin in solubility as well (BIETZ and WALL, 1973) (Fig. 9) : glutenin's subunits could be divided into two distinct fractions on the basis of solubility in neutral 70 p. 100 ethanol. The ethanol-insoluble fraction (38 p. 100) contained glutenin's characteristic high MW subunits,

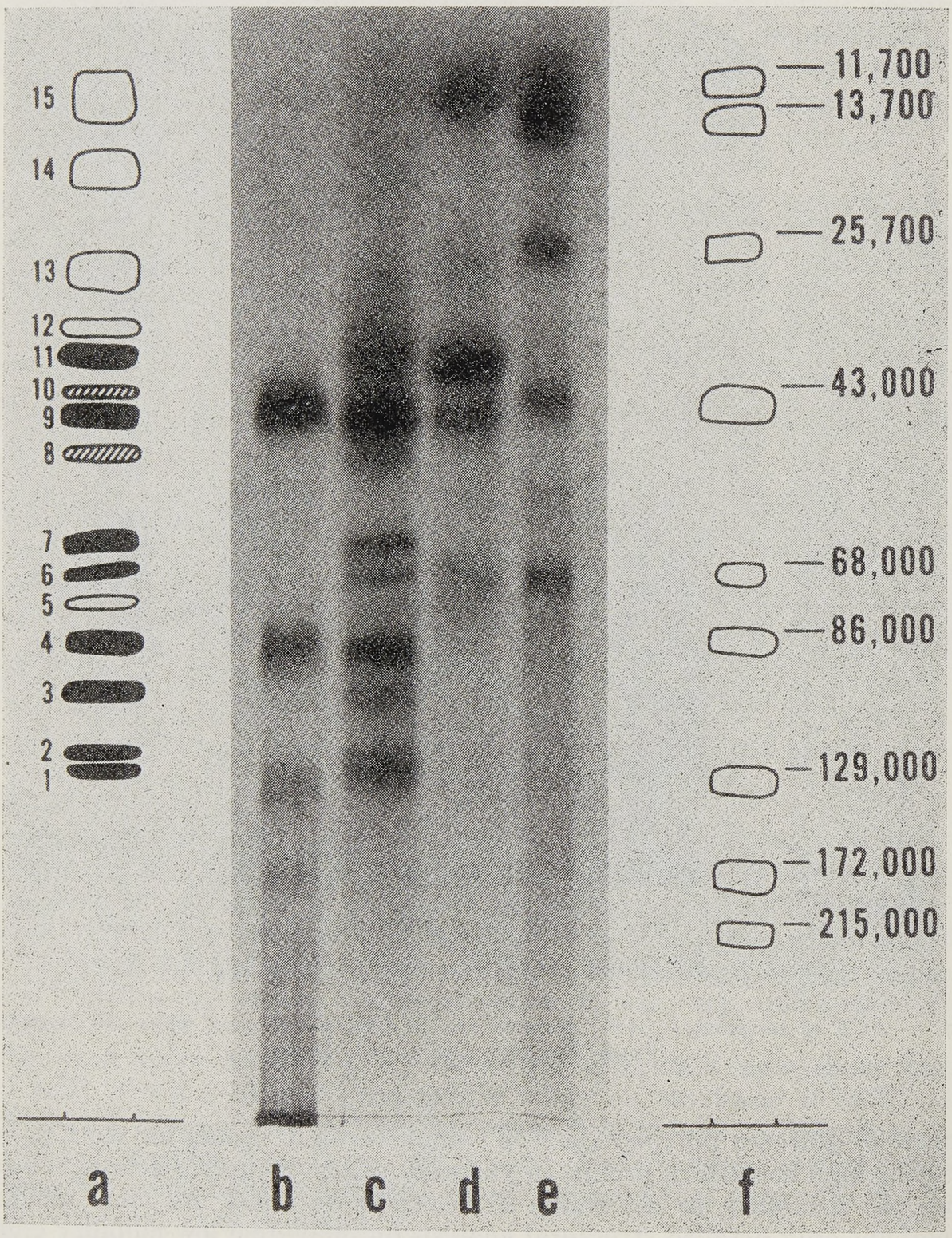

FIG. 8. — *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of glutenin (a, c), gliadin (d), and standard protein calibration mixtures (b, e, f).* (From BIETZ and WALL, 1972).

whereas the ethanol-soluble fraction (26 p. 100) was enriched in 44 000 and 36 000 MW subunits and was very similar to high MW gliadin (also called low MW glutenin) (NIELSEN *et al.*, 1968). Recently PAGE in 4M urea-pH 3.2 aluminum lactate buffer has demonstrated that ethanol-soluble and -insoluble glutenin each have at least 20-25 subunits, so glutenin contains at least 40-50 unique subunits (BIETZ, 1979). The relative amounts of ethanol-soluble and -insoluble glutenin subunits could influence its crosslinking, molecular size, and functional properties (BIETZ and WALL, 1973).

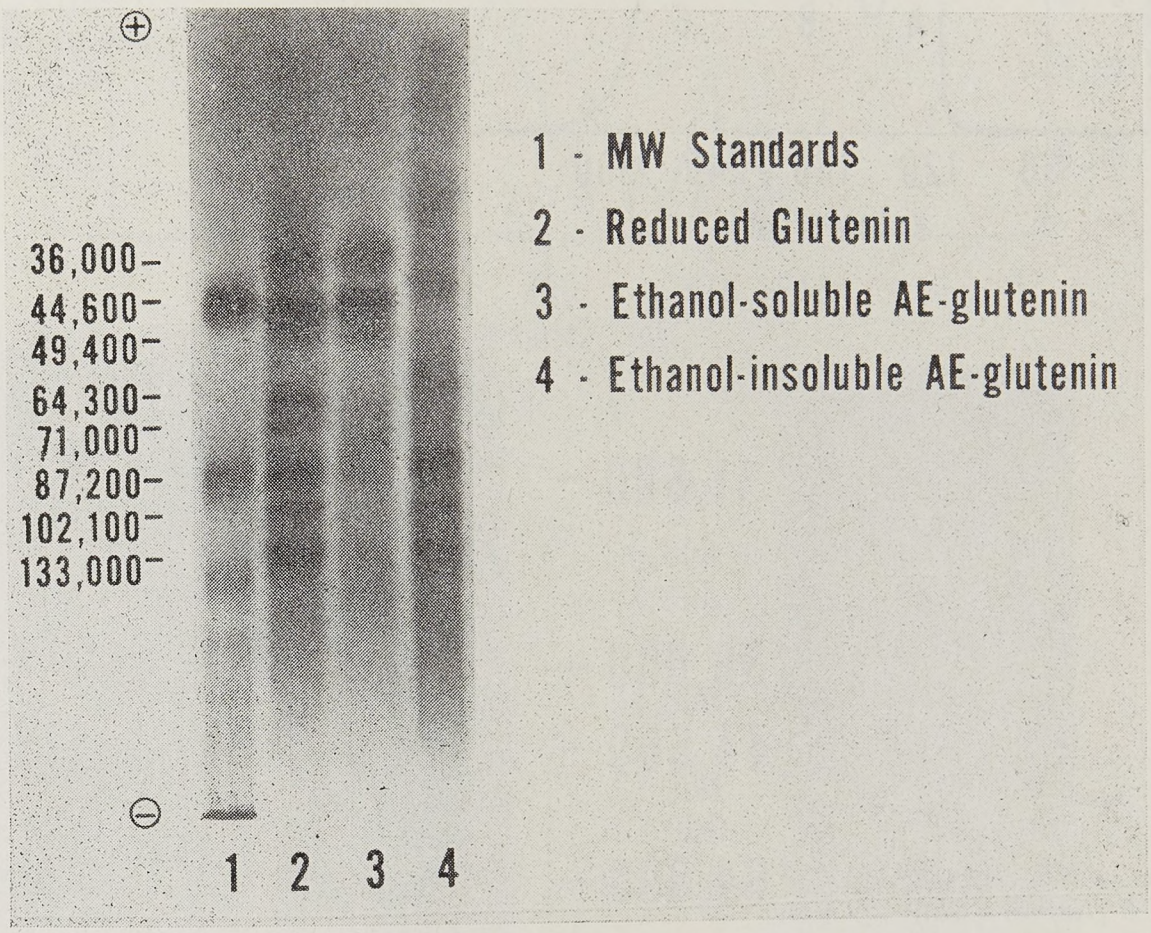

FIG. 9. — *SDS-PAGE of AE-glutenin (2) and its ethanol-soluble (3) and -insoluble (4) fractions. Molecular weight (MW) standards are shown in (1).* (From BIETZ and WALL, 1973).

In 1974, HUEBNER and WALL and HUEBNER *et al.* described the isolation and characterization of individual glutenin subunits. Gel filtration on Sephadex G-200 (Fig. 10) divided pyridylethyl- (PE-) glutenin into three peaks, each containing distinctly different subunits (Fig. 11). SDS-PAGE revealed that peak C consisted of 36 000-44 000 MW polypeptides resembling ethanol-soluble glutenin (BIETZ and WALL, 1973) or gliadin, peak B contained glutenin's high MW ethanol-insoluble subunits (BIETZ and WALL, 1973), and peak A consisted of lower MW proteins that are extensively aggregated, even in 4M urea. The high MW (B) and aggregated (A) subunits of glutenin may be essential to native glutenin's unusual characteristics. Further fractionation of subunits was possible by ion-exchange chromatography on sulfoethylcellulose (Fig. 12); for the first time, individual glutenin subunits were isolated. Characterization of these subunits in terms of amino acid composition and N-terminal amino acids revealed signi-

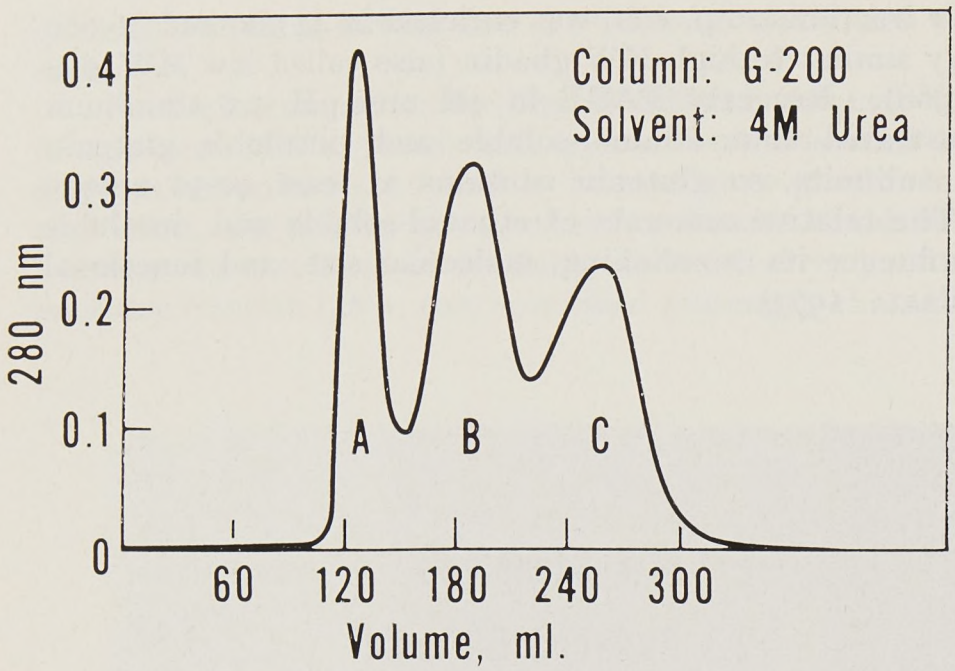

FIG. 10. — *Gel filtration of pyridylethyl- (PE-) glutenin on Sephadex G-200.* (From HUEBNER and WALL, 1974).

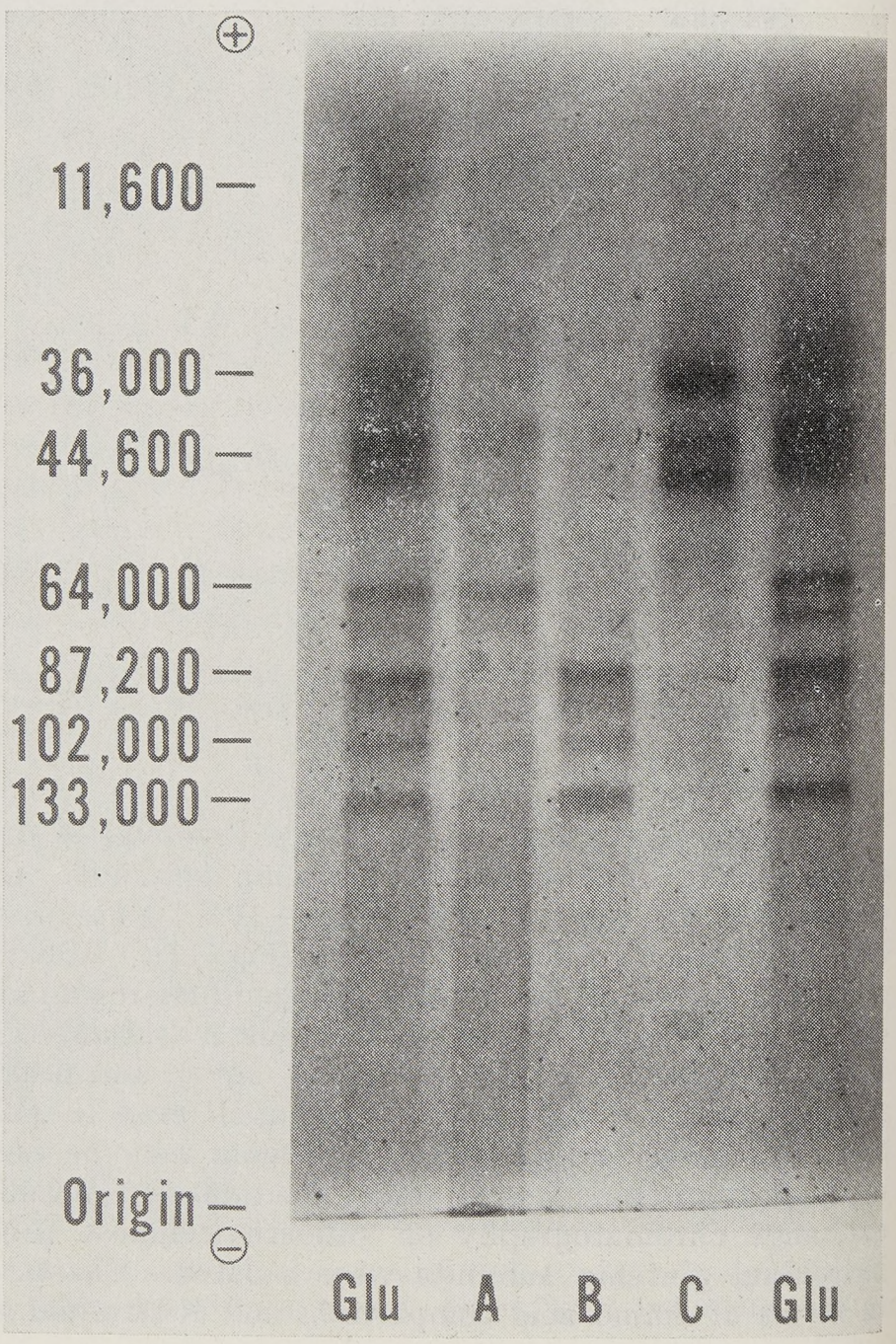

FIG. 11. — *SDS-PAGE of PE-glutenin (Glu) and its G-200 fractions* (Fig. 10). (From HUEBNER and WALL, 1974)

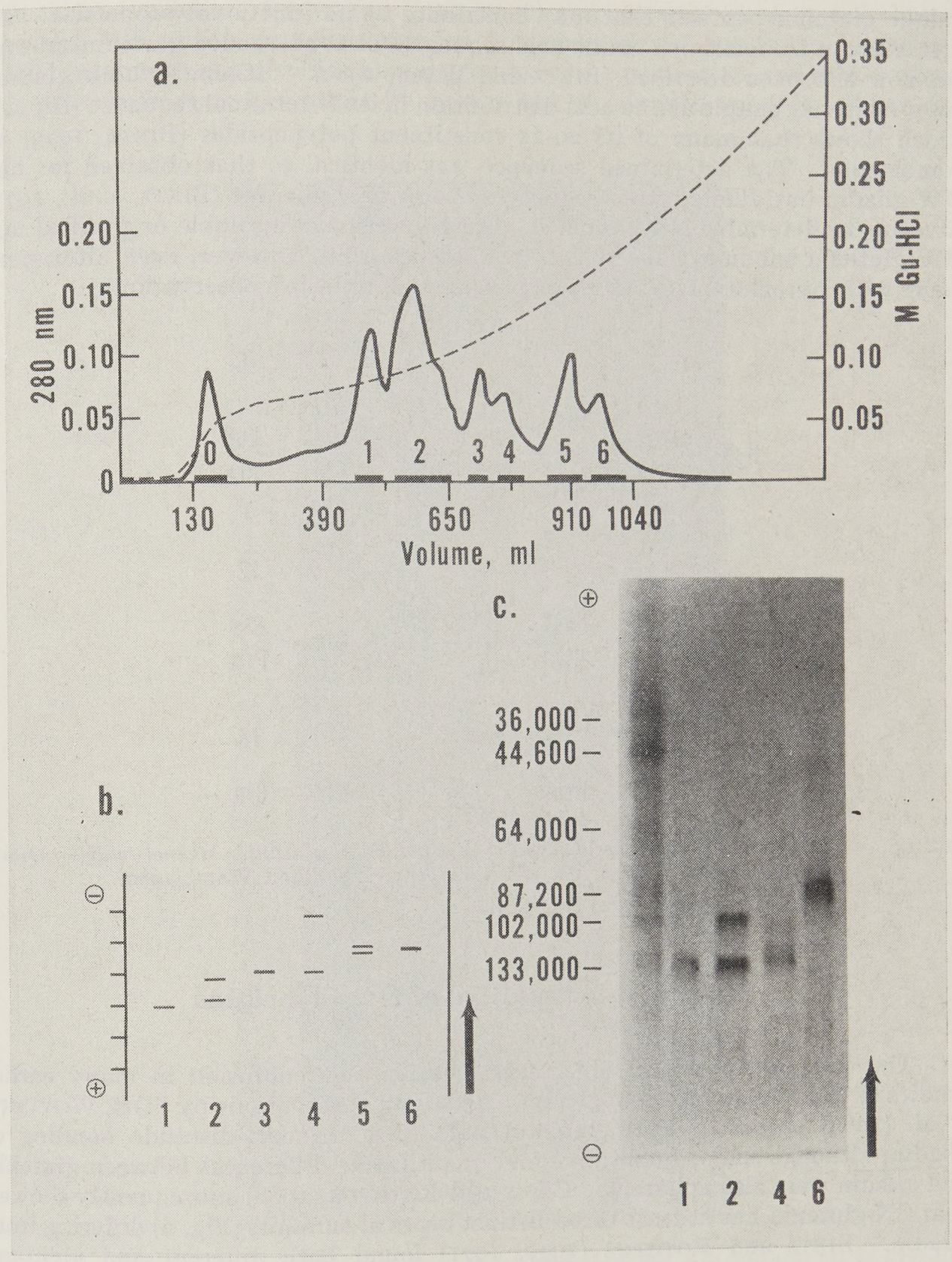

FIG. 12. — *Ion-exchange chromatography of fraction B of PE-glutenin obtained by Sephadex G-200 chromatography* (see Fig. 10) (a), *and analysis of fractions from* (*a*) *by SGE* (*b*) *and SDS-PAGE* (*c*). (From HUEBNER and WALL, 1974).

ficant differences that suggested different origins for the three types of glutenin subunits.

A basic goal of our research has been to relate wheat's functional properties to protein structures. BIETZ and ROTHFUS (1970, 1971) demonstrated that glutenin and gliadin are different in amino acid sequence, and they isolated some

unique glutenin peptides that may contribute to its functional properties. The first attempt to determine amino acid sequences of nondegraded glutenin subunits has now also been described (BIETZ and WALL, 1980). Ethanol-soluble glutenin has a relatively simple amino acid distribution in its N-terminal sequence (Fig. 13), which shows that many of its 20-25 constituent polypeptides (BIETZ, 1979) are homologous. The determined sequence was identical to that obtained for high MW gliadin but differed from sequences of low MW gliadins (BIETZ, *et al.*, 1977). Attemps to determine N-terminal sequences of ethanol-insoluble or purified high MW glutenin subunits have so far been unsuccessful, however, even after treatment with pyroglutamate amino peptidase (unpublished observation).

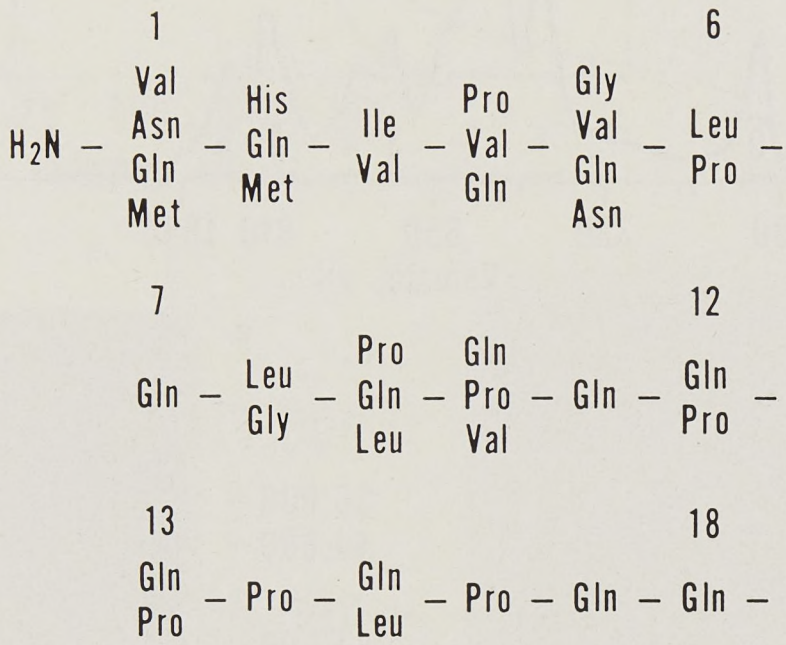

FIG. 13. — *Amino-terminal amino acid sequence distribution of glutenin's ethanol-soluble subunits and of subunits of high MW gliadin.* (From BIETZ and WALL, 1980).

Protein interrelationships and origins

The complexity of glutenin caused considerable confusion in many earlier studies as to its relationship to gliadin. Based on observations by SGE, WOYCHIK *et al.* (1964) suggested that glutenin could arise through disulfide bonding of gliadin, although major qualitative and quantitative differences between glutenin and gliadin were also apparent. CROW and ROTHFUS (1968) subsequently showed that CN-glutenin has at least three distinct types of subunits (Fig. 6) differing from gliadin. BIETZ and ROTHFUS (1970, 1971) found both different and identical peptides in proteolytic digest of gliadin and glutenin, suggesting that some polypeptides are unique to each protein class but others may be common to both, or may have a common origin. HUEBNER and ROTHFUS (1971) showed that glutenin is highly aggrageted, is incompletely extracted from flour with urea, and differs from gliadin. Much uncertainty as to the relationship of gliadin and glutenin was resolved by SDS-PAGE (BIETZ and WALL, 1972) (Fig. 8) : most glutenin subunits are uniquely different from gliadin, but some having MW's of 36 000-44 000 may be common to both protein classes.

Some of these 36 000-44 000 MW polypeptides are actually common to

glutenin and gliadin. Beckwith *et al.* (1966) and Nielsen *et al.* (1968) showed that high MW gliadin proteins excluded from Sephadex G-100 resemble glutenin in MW, intermolecular disulfide bonding, amino acid composition, and SGE pattern. This protein fraction has primarily 44 000 and 36 000 MW subunits (Bietz and

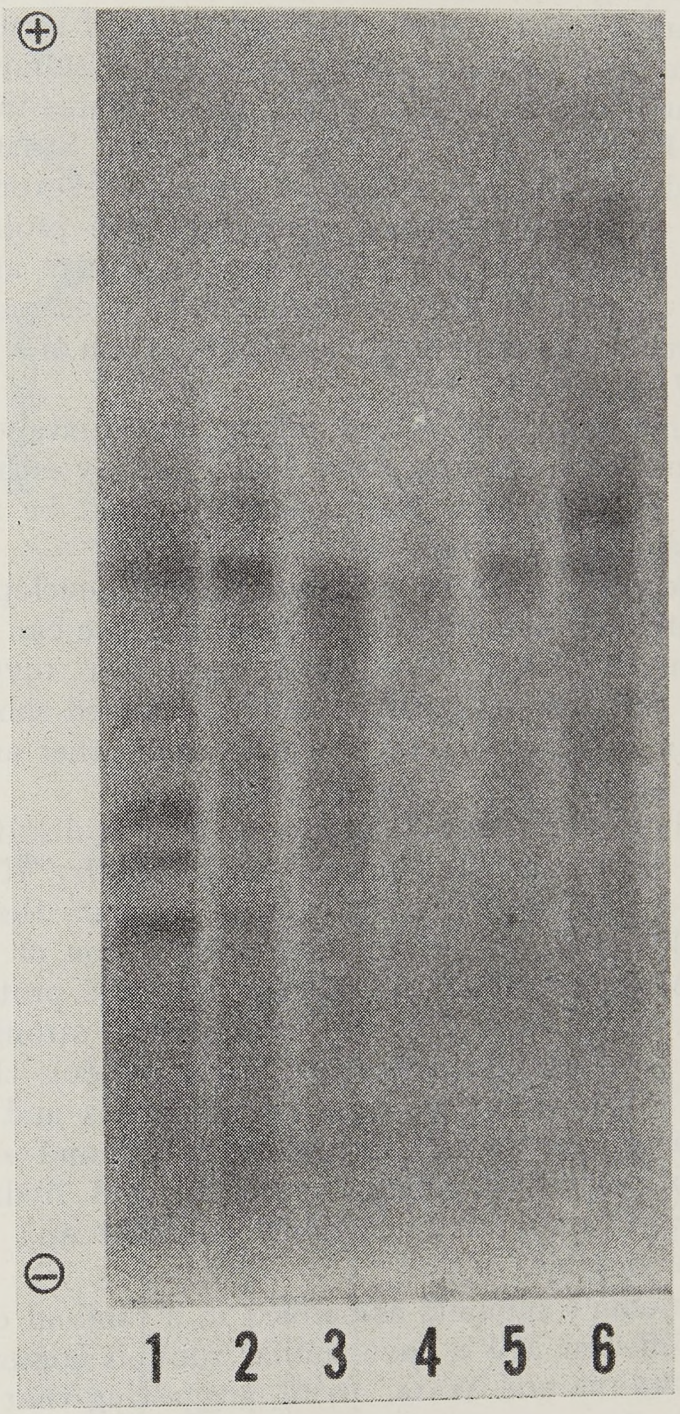

Fig. 14. — *SDS-PAGE patterns of* (1) *glutenin*, (2) *ethanol-soluble AE-glutenin*, (3) *44 000 MW subunit fraction of ethanol-soluble AE-glutenin*, (4) *44 000 MW subunit fraction of high MW gliadin*, (5) *high MW gliadin, and* (6) *gliadin.* (From Bietz and Wall, 1973).

Wall, 1972), similar to ethanol-soluble glutenin subunits (Fig. 14) (Bietz and Wall, 1973). High MW gliadin and ethanol-soluble reduced glutenin were also shown to have similar electrophoretic patterns and amino acid compositions (Bietz and Wall, 1973, 1980), and ultimately to have identical N-terminal sequences (Fig. 13) (Bietz and Wall, 1980).

Where do glutenin's subunits originate, and how are they joined together? CROW and ROTHFUS (1968) recognized that glutenin contains distincts types of subunits (Fig. 6) that could have different origins, and BIETZ and WALL (1973) suggested different origins for ethanol-soluble and -insoluble glutenin subunits. HUEBNER *et al.* (1974) suggested different origins for the three observed types of glutenin subunits (Fig. 10), since " A " subunits are highly aggregating, " B " subunits have an amino acid composition suggestive of structural proteins, and " C " subunits form both inter- and intra-molecular disulfide bonds rather than only intra-molecular bonds as in gliadin, which originates in protein bodies (see WALL, 1979*b*, and KASARDA *et al.*, 1976 for reviews). SIMMONDS (1972), meanwhile, also suggested that glutenin consists of proteins of several types and origins. All alcohol-soluble cereal polypeptides, including both prolamins and subunits of glutenins, mays originate in protein bodies (see BIETZ and WALL, 1980, for discussion). Some support for this possibility is that ethanol-soluble glutenins, like gliadins, are homologous, apparently due to mutation of a duplicated ancestral gene (BIETZ and WALL, 1980).

Glutenin subunits could be joined either immediately after synthesis or during kernel maturation or drying; they could join by either covalent or non-covalent bonds, or by both; and their assembly could be random or ordered. Each of these possibilities will now be considered.

High MW glutenin proteins exist early in kernel development (BUSHUK and WRIGLEY, 1971), but their subunit composition and size can vary due to mixing and dough formation (BIETZ *et al.*, 1975; BIETZ and WALL, 1975) or storage (HUEBNER, 1979). Thus, glutenin subunits probably are joined shortly after synthesis, but subsequent changes due to interaction with each other and with other endosperm proteins can occur.

Numerous studies have shown that intermolecular disulfide bonds maintain high MW glutenin structures, as recently confirmed by HUEBNER and WALL (1980). High-MW species were present even in solvents that disrupt hydrogen or hydrophobic bonds (Fig. 5), but the combined action of reducing agent and SDS liberates low MW subunits. In addition, low-MW protein (fraction "A " of HUEBNER *et al.*, 1974) is now known to associate strongly through hydrophobic bonds with glutenin (HUEBNER and WALL, 1980); this protein fraction may be important to glutenin's aggregating properties and ability to interact with protein such as gliadin (BIETZ and WALL, 1975) and α-amylase (ROTHFUS and KENNEL, 1970). Thus, disulfide, hydrogen, and hydrophobic bonds all contribute to glutenin's structure; additional covalent and non-covalent interactions may also be possible.

Finally, are glutenin's subunits assembled in a random or ordered manner? Some glutenins of different MW's have similar subunit compositions (CROW and ROTHFUS, 1968; HUEBNER and WALL, 1976), suggesting that they differ only in the degree to which the same subunits are repeated in their composition, and that intermolecular disulfide crosslinks are formed randomly *in situ*. Thus, residue protein may differ from soluble glutenins I and II (fig. 4) mainly in the extent of aggregation and crosslinking due to changes in proximity of different subunits at different moisture levels, stages of maturity, or environmental conditions. Glutenins of different solubilities do differ in subunit composition, however (BIETZ and WALL, 1975); since these differences seem to be constant for any variety, they suggest that some ordered assembly of glutenin subunits also exists. Since glutenin's " B " and " C " subunits (Fig. 10) can be separated only after reduction, it is also more likely that they are covalently linked by disulfides than

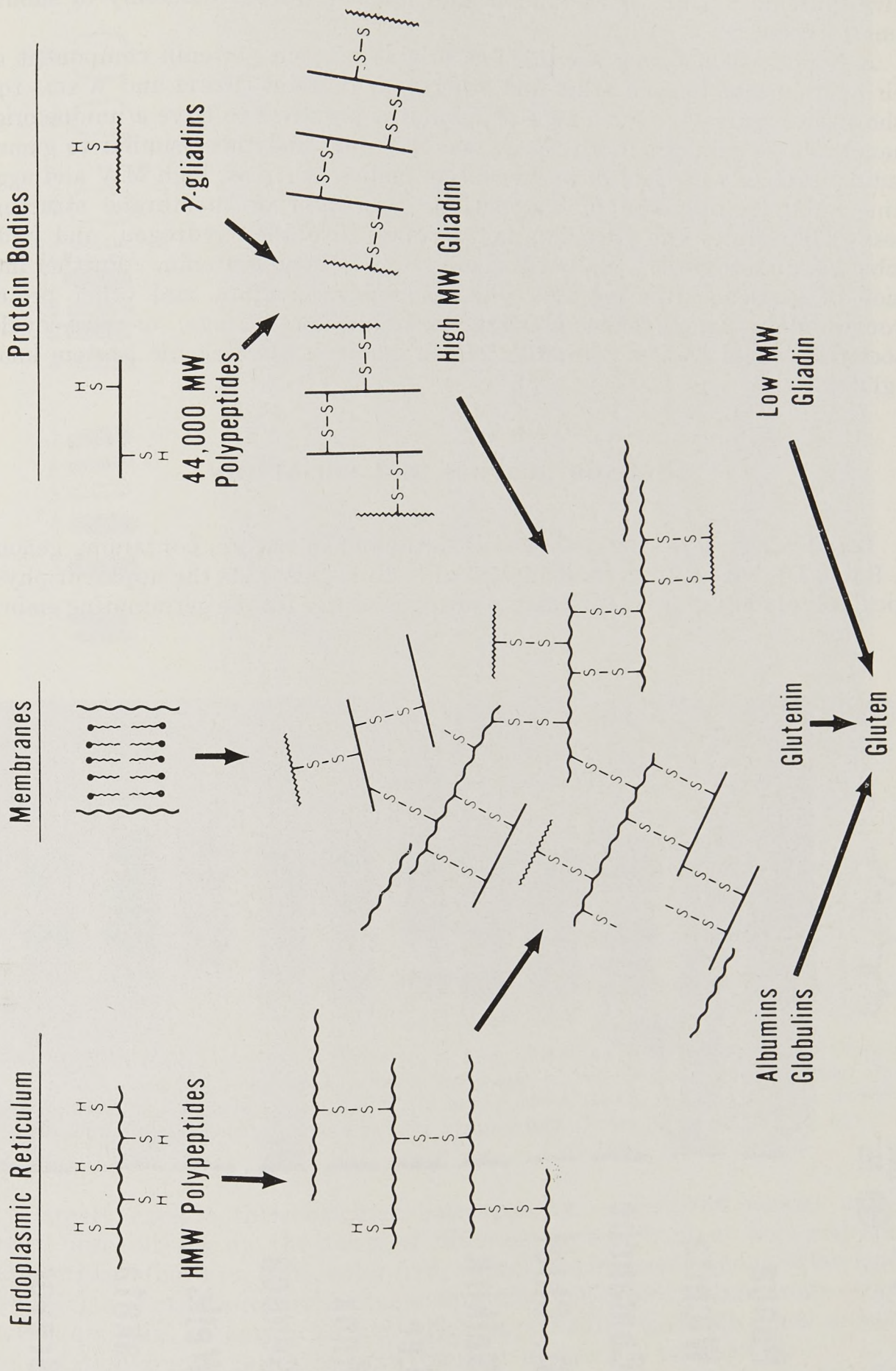

FIG. 15. — *Origins and interactions of wheat proteins.*
(After BIETZ and WALL, 1980)

that polypeptides of each type first join together and then are assembled into native glutenin. Thus, both random and mainly ordered assembly of subunits seems to occur.

A hypothesis showing the possible origins of each glutenin component and their relationships to each other and other flour proteins (BIETZ and WALL, 1980) is shown in Figure 15. Each type of subunit is proposed to have a unique origin: ethanol-soluble subunits (both the 44 000 MW ones and those similar to gamma-gliadins) probably are laid down in protein bodies, whereas, high MW and aggregating subunits originate in endoplasmic reticulum or membrane structures. These polypeptides can then interact through disulfide, hydrogen, and hydrophobic bonding to form " high MW gliadin " or to form glutenin. Further interaction of glutenin with low-MW gliadins, omega-gliadins, and other proteins through additional covalent bonding, disulfide interchange, or non-covalent association would lead to formation of the complex visco-elastic protein known as gluten.

Glutenin genetics and variability

Bread wheat, *Triticum aestivum*, is hexaploid in nature, containing genomes (A, B and D) derived from three diploid ancestors; this, plus the apparent physiological role of most gluten proteins as a nitrogen source for the germinating embryo,

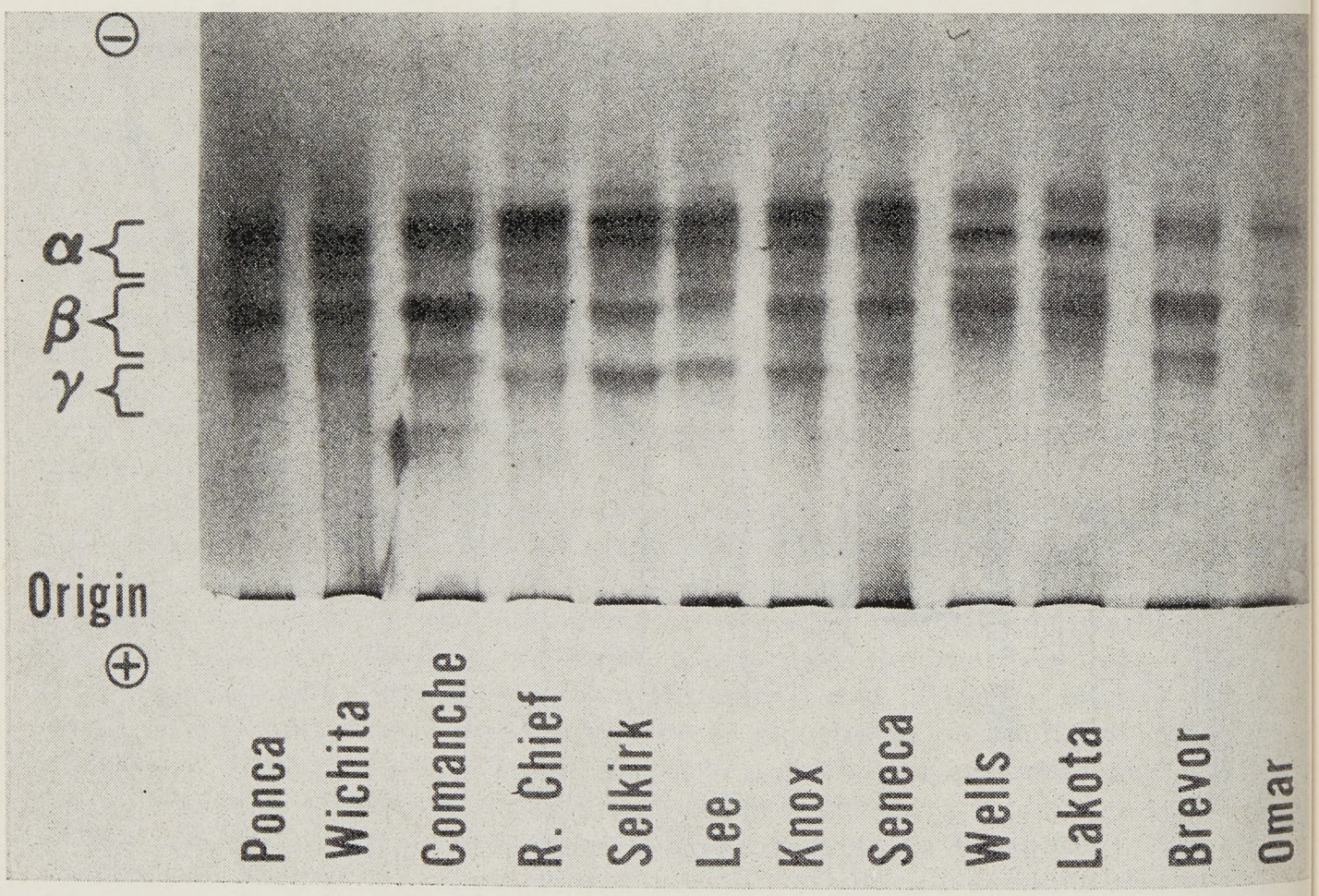

FIG. 16. — *SGE of reduced and alkylated glutenins of different classes and varieties.* (From HUEBNER, 1970)

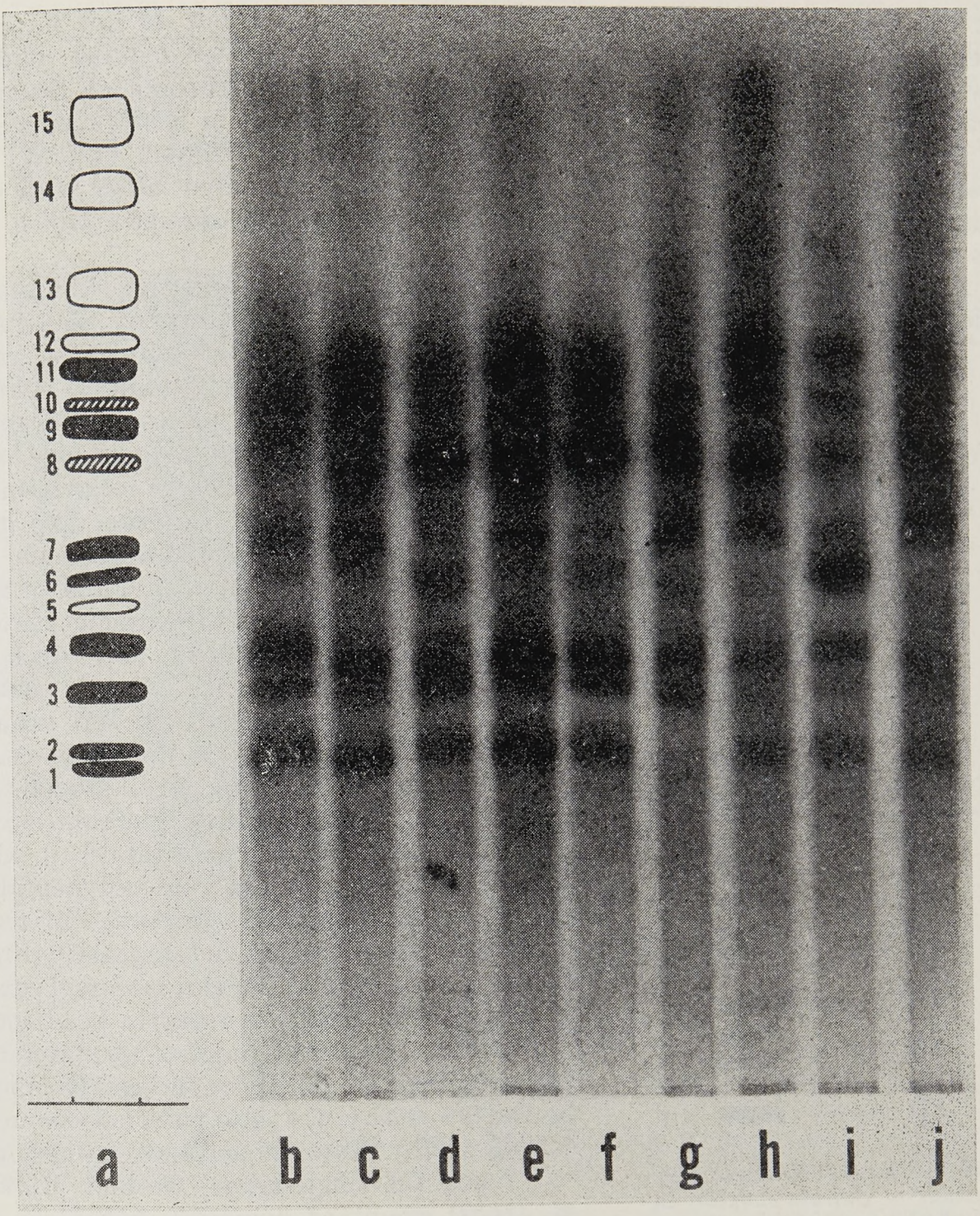

FIG. 17. — *SDS-PAGE of glutenins from* (*a*, *b*) *Ponca hard red winter wheat*, (*c*) *Red Chief hard red winter wheat*, (*d*) *Comanche hard red winter wheat*, (*e*) *Selkirk hard red spring wheat*, (*f*) *Red River 68 hard red spring wheat*, (*g*) *Wells durum wheat*, (*h*) *Omar club wheat*, (*i*) *Brevor soft white winter wheat, and* (*j*) *Seneca soft red winter wheat.* (From BIETZ and WALL, 1972).

may largely explain their extreme heterogeneity. Hexaploid wheats may be divided into classes on the basis of differing protein content and endosperm characteristics (hard *vs.* soft), color (red, white), environmental adaptation (spring *vs.* winter), and other properties; due to mutation and breeding, numerous varieties or cultivars exist within each class. Not surprisingly, qualitative and quantitative variation among glutenins from different wheats has been noted.

HUEBNER (1970) compared reduced and alkylated glutenins from 11 varieties (5 classes) of wheat by SGE and noted significant variations in subunit composition both within and between classes (Fig. 16); perhaps the greatest difference was

that the tetraploid durum wheats Wells and Lakota, which do not have the D-genome chromosomes of hexaploid wheat, lacked the slowest-moving subunits. This difference was particularly interesting since the D genome of hexaploid wheat seems responsible for gluten's functional properties in bread baking.

Bietz and Wall (1972) compared glutenins from different varieties and classes using SDS-PAGE (Fig. 17). Much similarity was noted, along with some distinctive differences. Durum wheat lacked two high MW subunits, which were thus tentatively associated with D-genome chromosomes.

A comprehensive study of the chromosomal control of glutenin subunits was made by examining aneuploid stocks, developed by E. R. Sears, by SDS-PAGE using single-kernel analysis (Bietz *et al.*, 1975). By comparing the parent variety, Chinese Spring, to compensating nullisomic-tetrasomic lines in which individual chromosome pairs had been substituted for by corresponding chromosomes from one of the other two genomes, the coding of five high MW glutenin subunits was established (Fig. 18). Three of these subunits were coded by D-genome chromosomes, including chromosome 1D, which has often been associated with wheat's quality characteristics; since these high MW subunits are unique to glutenin, they thus may contribute to its functional properties. Single-chromosome coding of most other glutenin electrophoretic bands could not be established, which suggests that more than one polypeptide is present in those bands, as confirmed by recent observations of glutenin's heterogeneity (Bietz, 1979). Knowledge of the chromosomal control of glutenin subunits has recently been confirmed and extended using higher-resolution one- and two-demensional electrophoresis techniques by Brown *et al.* (1979; also personal communication) and by Wrigley and Lawrence (1980).

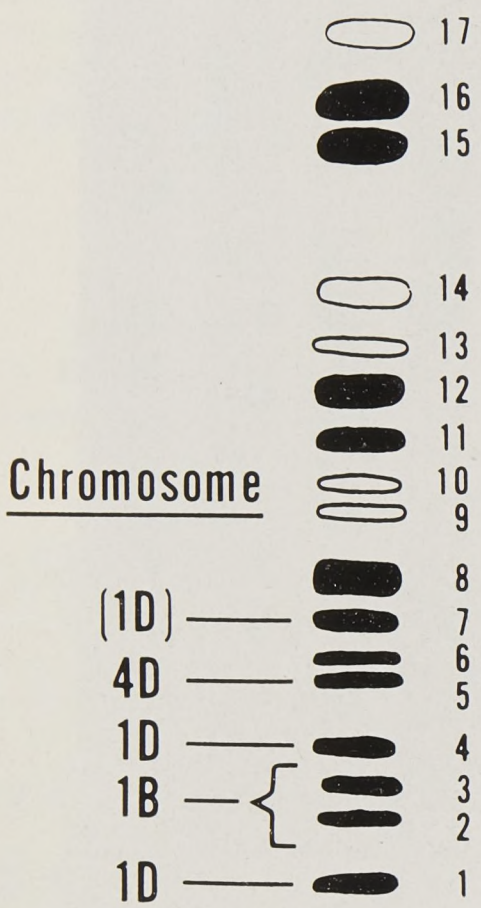

Fig. 18. — *Chromosomes controlling synthesis of glutenin subunits in hexaploid wheat.* (From Bietz, 1976)

Using the single-kernel method, Bietz *et al.* (1975) examined numerous hexaploid and durum wheat varieties. Of 80 hexaploid wheats of different origins and qualities, 75 had glutenin subunit compositions that were qualitatively and quantitatively very similar (Fig. 19). This suggested that high MW glutenin subunits were necessary but not sufficient for glutenin's functional properties. Five varieties had significantly different glutenin subunit compositions; these included the high-protein, high-lysine variety Nap Hal, which also contained different biotypes in a bulk sample.

Glutenins of 55 tetraploid wheats were also examined by SDS-PAGE (Bietz *et al.*, 1975) (Fig. 20). More variability in subunit composition was noted than for the hexaploid wheats (Fig. 19). These results suggest that the SDS-PAGE single-kernel technique can be valuable to breeders and geneticists in screening for unusual hexaploid and tetraploid genotypes having desirable characteristics, and for demonstrating homogeneity or heterogeneity of bulk samples.

Knowledge of polypeptide coding permits using electrophoretic bands as markers of characteristics associated with individual chromosomes. For example, JOPPA *et al.* (1975, 1979) showed that SDS-PAGE of glutenins from durum aneuploids is valuable for selection and chromosomal identification. A disomic 1D addition line to Langdon durum contains two additional high MW glutenin subunits (Fig. 21) and also has improved protein content and mixing characteristics

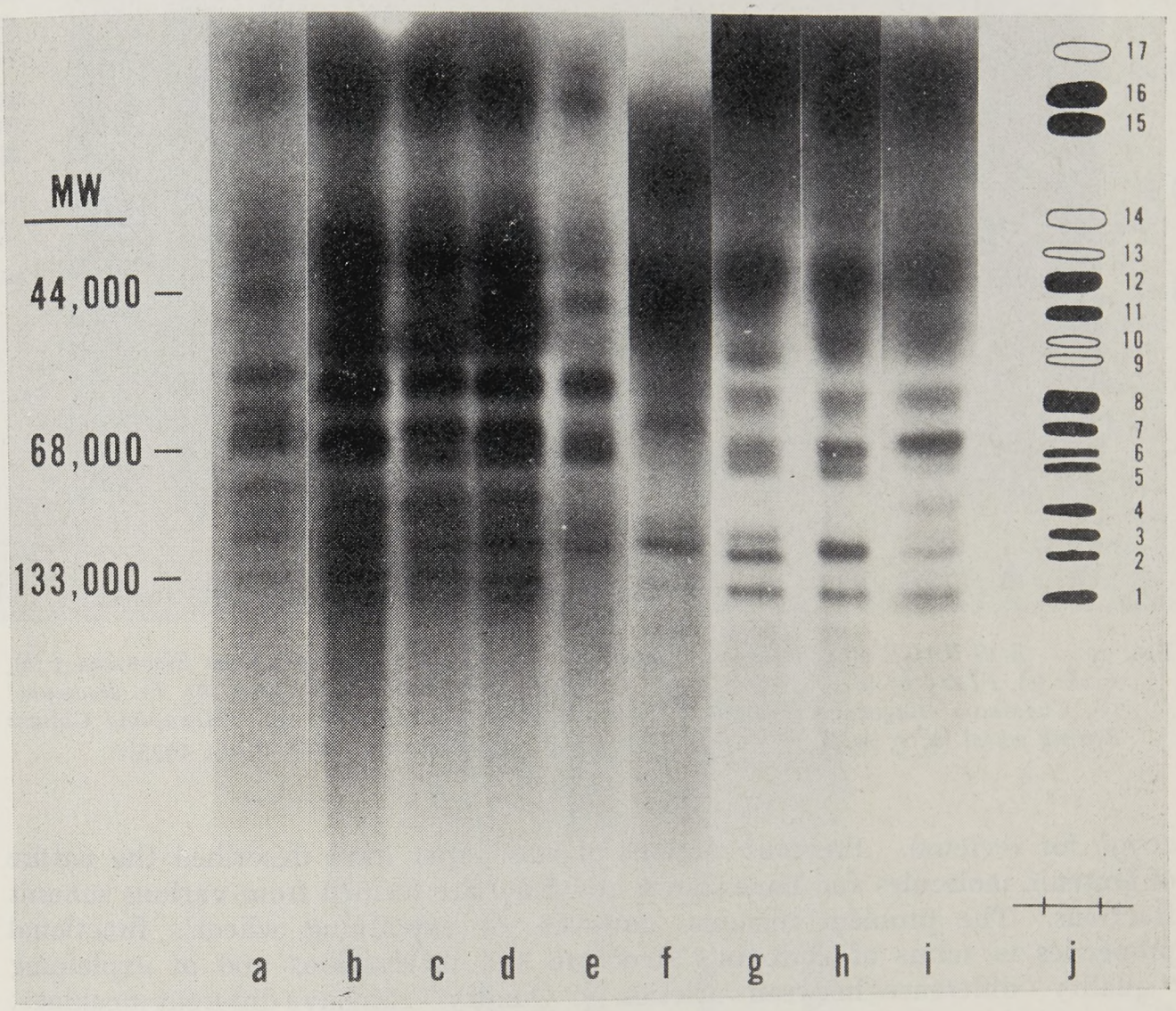

FIG. 19. — *SDS-PAGE of glutenins from single kernels of the hexaploid wheats Chinese Spring (a, j), Vulcano (b), MV-72-16 (c), Hokuei (d), Nap Hal (e), and unusual Nap Hal genotypes (f-i).* (From BIETZ *et al.*, 1975).

that may be related to these subunits; alternatively, these subunits are excellent markers of other quality characteristics on the 1D chromosomes.

Glutenin quality and functionality

Hydrated glutenin is cohesive and elastic in nature and is a major contributor to wheat's functional properties (see BIETZ *et al.*, 1973; WALL and BECKWITH, 1969; KHAN and BUSHUK, 1978; HUEBNER, 1977 and 1978; and WALL 1967 and

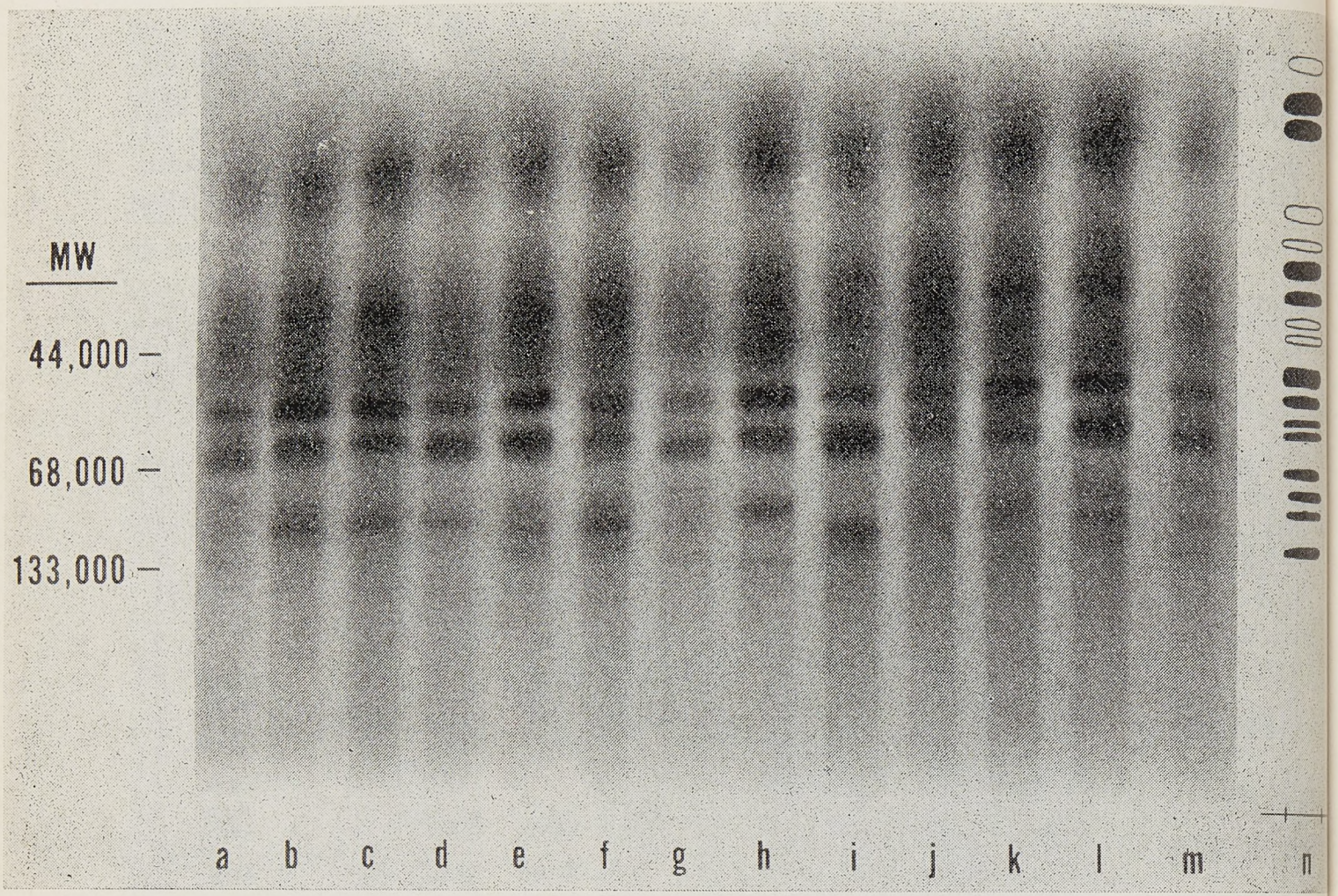

FIG. 20. — *SDS-PAGE of glutenins from single kernels of the tetraploid wheats Sivousska-3 (b), Leeds (c), PI225167 (d), Tr. turgidum (e), St 464 (f), Bagudo 10150 V56A (h), Tr. dicoccum? (i), Cudesnaja Blagodat (j), Tremes Preto 3687 (k), and Argelino (1). Hexaploid Chinese Spring wheat (a, g, m, n) is included for comparison.* (From BIETZ *et al.*, 1975).

1979*b* for reviews). Previous sections of this paper have described the nature of glutenin molecules and have shown how they are formed from various subunit fractions. The problem remains, however, of explaining wheat's functional properties in terms of glutenin's structure and interactions and of explaining " quality " differences between varieties. " Quality " can have different meanings; for most baking applications, however, a " good-quality " wheat will have sufficient protein, of the right type, to form a uniform and reasonably stable dough network under normal mixing conditions; this protein must also have elastic properties that impart an acceptable volume to the finished loaf.

Since wheat varieties do differ greatly in quality, and since glutenins of most wheats have very similar overall qualitative and quantitative subunit composition (Fig. 19) (BIETZ *et al.*, 1975), other factors must link glutenin structure and wheat quality. The size distribution of native glutenin molecules seems to be this link, as demonstrated by studies at NRRC that agree with results of many other investigators (see BIETZ *et al.*, 1973; KHAN and BUSHUK, 1978; and WALL, 1979*b* for reviews).

HUEBNER (1970) showed that glutenins from 11 wheat varieties and classes of different qualities differed in their responses to salt precipitation; this suggests that gluten quality was related to sensitivity of glutenin to changes in ionic strength. Glutenins from good varieties had steep precipitation curves, suggesting

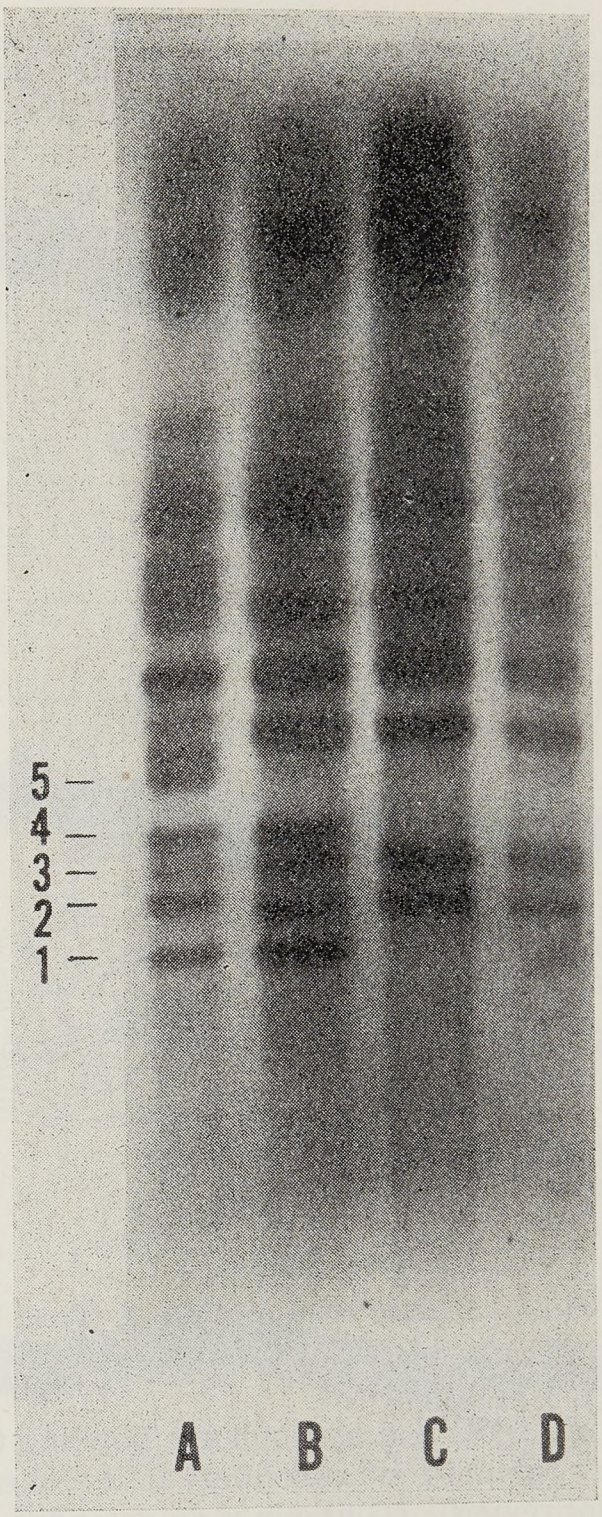

FIG. 21. — *SDS-PAGE of glutenins from (a) Chinese Spring hexaploid wheat (b) disomic-1D addition line to Langdon durum, (c) Langdon durum wheat, and (d) 28-chromosome sister line of (b) lacking 1D chromosomes.* (From JOPPA *et al.*, 1975).

that they had a higher MW distribution and were more easily salted out of solution. HUEBNER and WALL (1976) also demonstrated that glutens from wheats of differing quality varied in their contents of Glutenin I and II (Fig. 4) and residue protein : strong, long-mixing flours had a high Glutenin I/Glutenin II ratio and contained much residue protein. These results indicated that sufficient glutenin is necessary to form a strong dough, but a proper proportion of all gluten molecular species is also essential. Recently HUEBNER (1979) has also shown that changes in glutenin's MW distribution occur during storage of whole wheat,

while baking quality is changing. These and other studies all indicate that wheat's quality is associated with native glutenin's size distribution: it is directly related to the amount of high MW, highly aggregated glutenin or insoluble residue protein.

Thus, glutenins are high MW proteins of varying sizes containing several type of subunits joined together both covalently and non-covalently. An appropriate ratio of intra-/inter-molecular disulfide bonding seems necessary for viscoelasticity (BECKWITH and WALL, 1966), but hydrogen bonding through amide

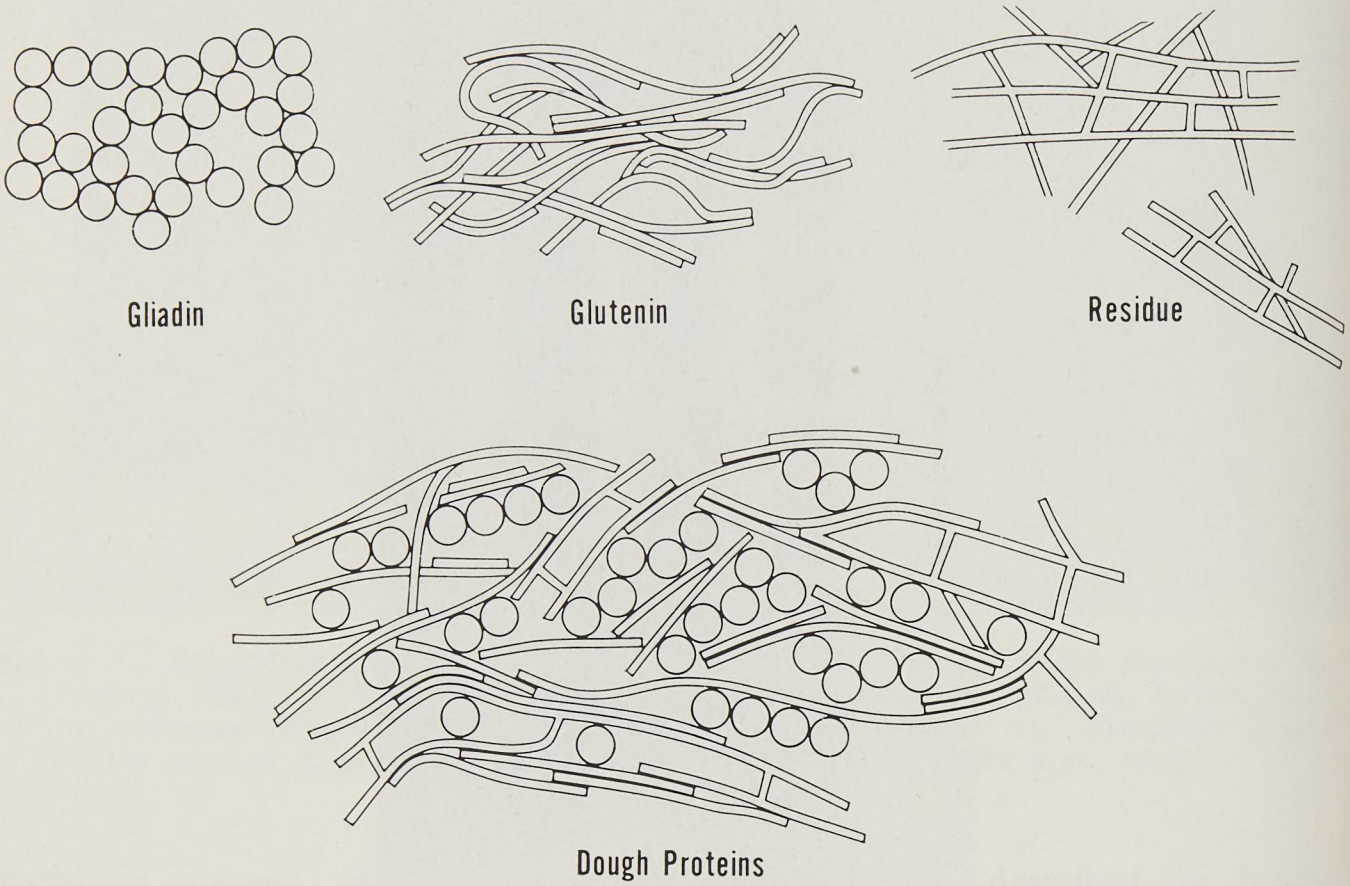

FIG. 22. — *Interactions of wheat proteins in dough.* (From WALL, 1979*b*).

residues (BECKWITH *et al.*, 1963) and hydrophobic bonding (HUEBNER and WALL, 1980) also join glutenin's subunits and contribute to its cohesive/elastic properties (see also WALL, 1979*a* for review). Glutenin is an extended, asymmetric molecule having high surface area due to its largely random structure; this promotes noncovalent association with other proteins and flour constituents. Because of its extended molecular structure, hydrated glutenin can form films (WALL and BECKWITH, 1969); as molecules become oriented, their cohesive interactions increase. This also seems to occur during dough formation (Fig. 22): mixing, disulfide interchange, and noncovalent interactions transform glutenin into a fairly linear, extended gluten network in which other flour constituents are dispersed. Many further reactions are also possible during mixing: oxidizing or reducing agents can promote or inhibit extension of the gluten network through disulfide interchange, and non-covalent interactions with various improvers, lipids, carbohydrates, and lower-MW proteins, such as gliadin, can occur. HUEBNER and WALL (1979) noted that certain polyaccharides change mixing time and dough stability (Fig. 23) and can actually improve weak flours. These poly-

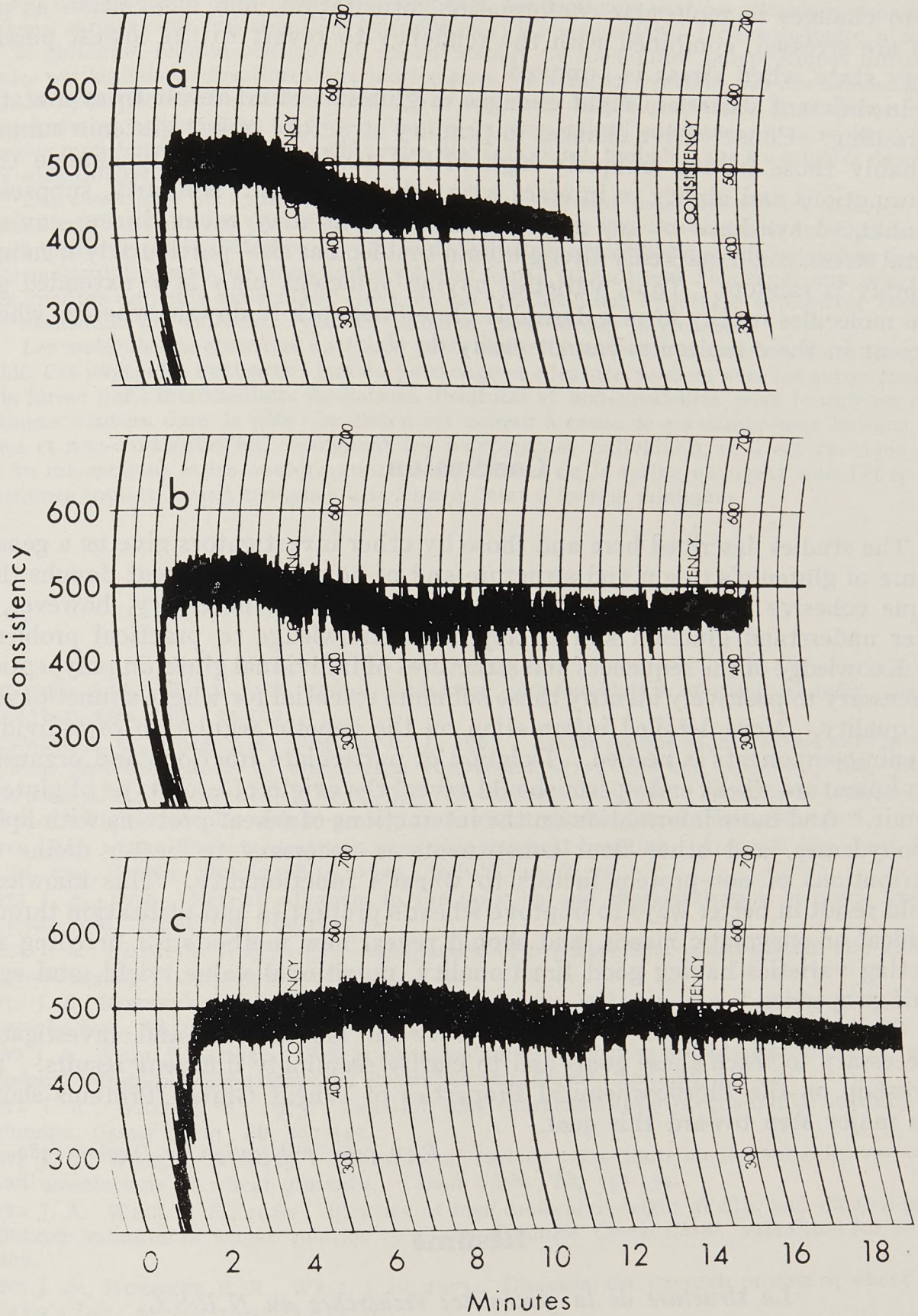

FIG. 23. — *Farinograph curves of standard soft red winter wheat flour (a) showing effect of addition of polysaccharide B-1459 (b) and calcium carrageenan (c).* (From HUEBNER and WALL, 1979).

accharides react specifically but non-covalently with gluten proteins, promoting formation of an extended gluten network similar to that which forms spontaneously in doughs from stronger wheats. The gluten network is in a minimum energy state, and gluten's elastic nature can thus be visualized as an extension

due to changes in molecular conformation, orientation, and association as proteins are stressed, combined with the tendency to revert to the lowest possible energy state when stress is removed.

In different varieties, slight changes in glutenin could result from mutation or breeding. Conservative changes in primary structure of key glutenin subunits (probably those having uniquely high MW's) could significantly change their conformations and ability to interact with other proteins. Similarly, suppressed or enhanced synthesis of key glutenin subunits, as may occur during environmental stress, could markedly alter glutenin's molecular size, particularly if subunit assembly is random. Thus, varieties having sufficient high MW extended glutenin molecules readily form a desirable gluten network in dough, whereas wheats deficient in these molecules cannot easily do so.

Conclusion

The studies described here and those by other investigators give us a general picture of glutenin's origin and structure and of how it gives wheat doughs their unique cohesive/elastic nature. Additional studies are necessary, however, to better understand glutenin and to apply our knowledge to practical problems.

Knowledge of the sequences and structures of individual glutenin polypeptides is necessary to positively identify those subunits essential for wheat's functionality and quality. More detailed information on the genetic regulation of individual glutenin components is needed. Isolation of particulate fractions and organelles from immature wheat endosperm should reveal the origin of each type of glutenin subunit. And more information on the interactions of wheat proteins with lipids, carbohydrates, and other flour constituents is necessary to better define the contributions of non-protein factors to wheat's functionality. This knowledge should result in better ways to improve wheat's properties and utilization through chemical or enzymatic means, and should reveal new methods for breeding and selecting varieties having good functionality, nutritional value, yield, and agronomic properties.

Obviously, continued cooperation between laboratories and investigators is necessary to reach these goals and to clarify seemingly different results. The Workshop on the Physicochemical Properties of Wheat Gluten Proteins should be a major step toward this goal.

Reçu pour publication en Octobre 1980.

Résumé

La structure de la gluténine: recherches du N.R.S.C.

Le Centre de Recherche de la Région du Nord (NRRC) qui dépend de l'Administration de la Science et de l'Éducation du Ministère de l'Agriculture des U.S.A., est situé à Péoria, Ill.

Depuis plus de 20 ans, des études ont porté sur la nature du gluten du blé et sur ses fractions principales, la gliadine et la gluténine. Cet aperçu résume les études faites sur la glutenine au NRRC et tente de faire la synthèse pour montrer quel est le rapport entre la structure de la gluténine et les propriétés fonctionnelles du blé.

Les premières études du NRRC ont établi des méthodes pour isoler la gluténine et ont démontré que c'est un mélange hétérogène de molécules de poids moléculaire élevé, qui contient

à la fois des structures ordonnées et désordonnées. Les molécules sont asymétriques et possèdent de larges surfaces qui favorisent les interactions et les associations. L'électrophorèse a montré que la gluténine réduite contient un grand nombre de sous-unités polypeptiques différentes, réunies par les liaisons disulfures intermoléculaires. On a mis en évidence que des liaisons hydrogènes et hydrophobes contribuent aussi à leur structure. Des sous-unités de la gluténine ont été fractionnées et caractérisées et l'on a montré qu'elles étaient constituées d'au moins 3 types, avec des propriétés et un poids moléculaire distincts; il est probable que les origines de chaque type sont différentes.

Des variétés de blé ayant des qualités différentes se sont montrées différer dans leur composition en sous-unités et on a établi le contrôle génétique des sous-unités de haut poids moléculaire particuliers à la gluténine. La gluténine se distingue singulièrement de la gliadine de faible poids moléculaire, mais ses sous-unités solubles dans l'éthanol sont identiques aux sous-unités de la gliadine de poids moléculaire élevé, ce qui conduit à une hypothèse expliquant comment sont rassemblés les différents types de protéines pour former la gluténine.

Les molécules de gluténine native de poids moléculaire élevé sont en relation avec la qualité du blé. Ces molécules s'orientent lors du pétrissage et elles interagissent avec les autres protéines de la farine par l'intermédiaire de liaisons disulfures et non-covalentes pour fournir un réseau glutineux continu dans la pâte. Ce gluten est cohésif à cause de ses nombreuses liaisons covalentes et non-covalentes qui rejoignent les polypeptides individuels, et il est élastique parce que les interactions et les conformations des protéines de la farine changent sous l'effet de la contrainte tout en ayant tendance à revenir à l'état d'énergie minimum.

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Functional glutenin : a complex of covalently and non-covalently linked components

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Summary

Glutenin is the protein of wheat flour that remains in the insoluble residue after sequential extraction with 0.5N sodium chloride and 70 p. 100 ethanol solutions. It constitutes 35-40 p. 100 of the flour protein and contributes elasticity to flour-water doughs and wet gluten. It contributes substantially to " breadmaking quality " and is responsible for most of the genotypic differences in breadmaking quality between cultivars of the same class of wheat. Considerable progress has been made on the structure of glutenin, in relation to functionality, especially through the application of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE results indicate that reduced glutenin comprises approximately 17 subunits ranging in molecular weight from 12 000 to 134 000. Solubility studies with amphiphilc (e.g. sodium stearate) and dissociating reagents (e.g. acetic acid-urea-cetyltrimethylammonium bromide) indicate that hydrophobic interactions play an important role in the insolubility and rheological stability of the functional glutenin complex. While some of the subunits can be liberated (solubilized) from the complex by the use of amphiphilic reagents, complete disruption of the complex into subunits requires the reduction of disulfide bonds. A close association has been demonstrated between a carbohydrate and some high molecular weight glutenin subunits. Preliminary results indicate that the carbohydrate moiety is covalently linked to the protein. The glycoprotein appears to be involved in the aggregation of specific glutenin subunits into large complexes. Qualitative analysis of the constituent sugars of the carbohydrate moiety indicates the presence of both pentoses and hexoses. These studies have led to a new model for the functional glutenin in which most of the subunits are held together and within a disulfide-crosslinked matrix by secondary forces (hydrophobic and ionic interactions, hydrogen bonds). The matrix, which comprises mainly the high molecular weight subunits, forms the structural framework of the functional glutenin complex.

Key words : *wheat — glutenin — composition — structure.*

Introduction

Among the many components of wheat flour that contribute to its inherent end-use quality, the main one is its protein. The protein component is important from two aspects. First is the nutritional quality of the final product, and second

is the ability of the flour to produce a satisfactory product with regard to aesthetic and organoleptic qualities. In relation to both aspects, the role of the protein component can be considered on the basis of two distinct parameters. First is the quantity of protein in the flour. This can be measured quite precisely by several different methods of analysis. The second parameter is the so-called " quality " of the protein. The definition of this parameter depends on whether the final product is assessed for nutritional or organoleptic quality. Nutritional quality of a protein can be assessed relatively easily; it depends mainly on the content and availability of the essential amino acids. On the other hand, organoleptic quality, (e. g. breadmaking quality) is much more difficult to measure

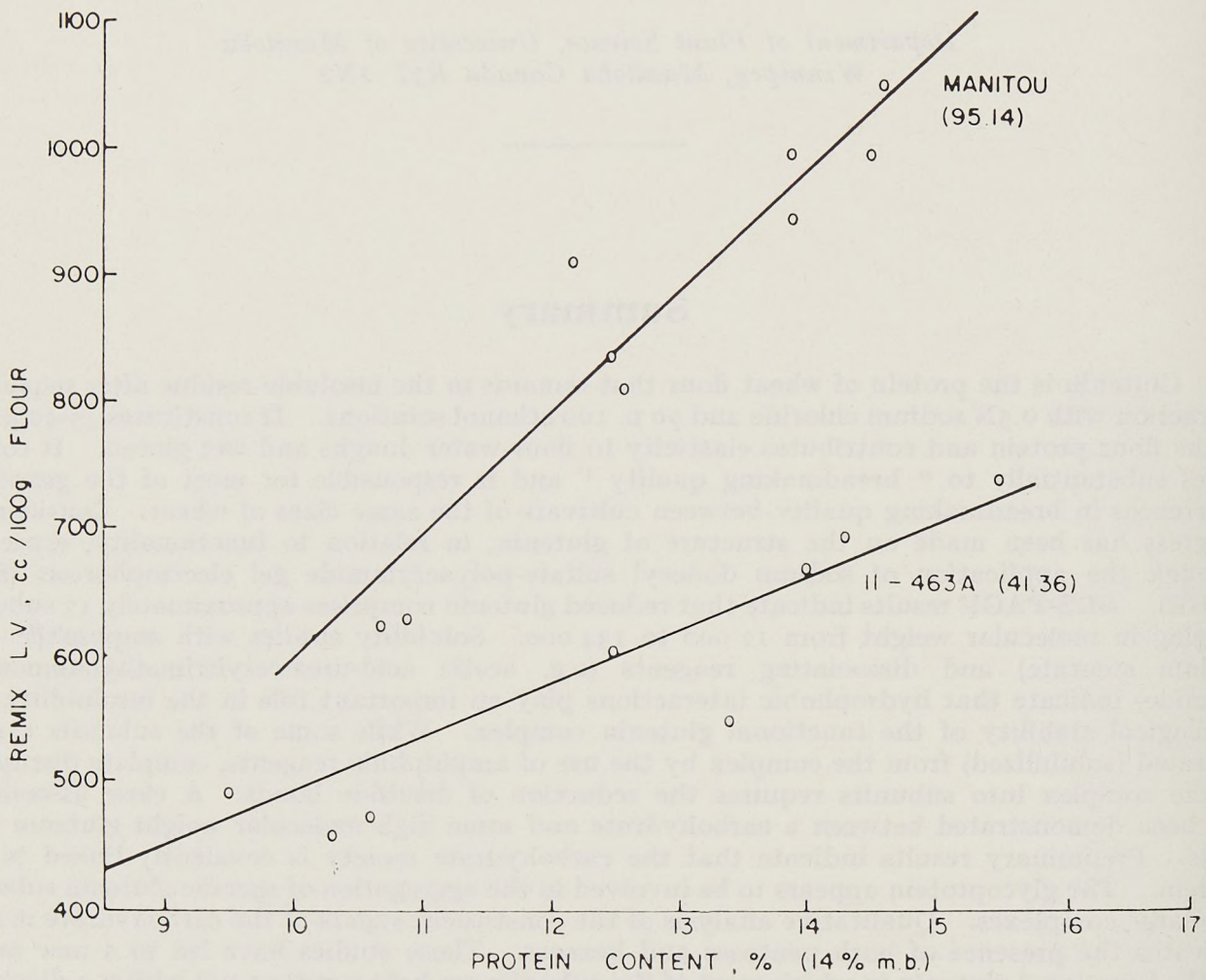

FIG. 1. — *Loaf volume as a function of protein content of flour milled from samples of two varieties of the hard red spring wheat class. The number in parentheses is the slope or increment of loaf volume for each unit of protein.*

objectively since it depends on a myriad of compositional, ingredient and processing factors.

The influence of both protein quality and quantity on breadmaking quality is clearly demonstrated in Figure 1 (BUSHUK *et al.*, 1969). As the protein quantity increases for both cultivars, Manitou and 11463-A, the loaf volume also increases. Loaf volume is considered to be the most accurate single index of expressing breadmaking quality. However, it is obvious there is a second factor, generally referred to as " protein quality ", and directly related to the slope of the lines in Figure 1, that distinguishes good quality cultivars from poor ones. In Figure 1, Manitou, at equivalent protein content, gives a much larger loaf volume than

11463-A. The quest for the fundamental definition of protein quality on the basis of chemical and physical properties of flour proteins has occupied many cereal scientists for almost a century.

One approach to the study of the nature of protein quality has been to fractionate flour proteins into five major groups on the basis of solubility in different solvents (OSBORNE, 1908). Earlier work from our laboratory (ORTH and BUSHUK, 1972) showed that of all the Osborne fractions, the amount of soluble glutenin is negatively correlated whereas the amount of residue protein is positively correlated to breadmaking quality. These results, based on 26 varieties of the hard red spring class, showed that the " quality " factor seems to be in the glutenin fraction of the flour protein. Accordingly, the search for a molecular basis of protein " quality " for breadmaking, in our laboratory, shifted, about 10 years ago, to intensive research on the glutenin fraction. In this paper we shall place major emphasis on the results obtained in our laboratory. The results indicate that protein complexing (aggregation/association) in the glutenin fraction plays a major role in influencing breadmaking quality. Protein complexing, therefore, will be the major theme of this article. Wherever possible, research by other workers that supports this concept will be cited accordingly.

Biosynthesis and storage of endosperm proteins

It is now quite certain that the aggregation (association) tendency of certain wheat proteins that is essential for functionality in breadmaking is a genotypic trait and not an artifact produced by the desiccation of the wheat grain at maturity or by the isolation procedures employed in the preparation of protein fractions for further study. Chemical and physical properties essential for aggregation are incorporated into the molecules during biosynthesis. Perhaps, it is the qualitative and quantitative differences in these aggregative properties that distinguish good from poor breadmaking quality cultivars.

Studies on developing grain have shown that glutenin is synthesized and deposited in the endosperm as a storage protein at very early stages of grain development. JENNINGS and MORTON (1963) and GRAHAM *et al.* (1963*a*) showed that storage protein (acetic acid-soluble) continued to increase from about day 15 (after anthesis or flowering) to maturity whereas the cytoplasmic protein (pyrophosphate buffer-soluble) increased only slightly from day 25 to maturity.

Studies on developing wheat grain with the electron microscope (GRAHAM, *et al.*, 1962) showed that the storage proteins were deposited in the endosperm in distinct protein bodies. These protein bodies have been isolated from immature endosperm and shown to contain slow moving protein components on starch-gel electrophoresis similar to the slow moving components of the acetic acid-soluble glutenin (GRAHAM *et al.*, 1963*b*). GRAHAM *et al.* (1962) postulated that the storage proteins were formed on ribosomes of the endoplasmic reticulum, then secreted internally, and finally aggregated into protein bodies within a surrounding lipoprotein membrane. SIMMONDS (1972) also observed protein bodies in an area of endosperm rich in endoplasmic reticulum. However, his electron micrographs did not show a distinct membrane surrounding the protein bodies.

Gel-filtration studies (BUSHUK and WRIGLEY, 1971) and studies by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (KHAN and BUSHUK, 1976) of the proteins of developing wheat grain also showed the presence

of glutenin at very early stages of grain development. In a recent study of compositional differences in developing grain of two Australian wheat cultivars, one of high and the other of low protein content, DONOVAN *et al.*, (1977*a*) found that the proportion of storage protein relative to cytoplasmic protein in each cultivar increased as development advanced. They also observed that the high protein cultivar contained a larger non-protein nitrogen pool and a greater protein synthetic capacity than the low protein cultivar (DONOVAN *et al.*, 1977*a*,*b*). It seems, therefore, that variation in the amount of acetic acid-soluble and residue protein among wheat cultivars, observed by ORTH and BUSHUK (1972) and ORTH and O'BRIEN (1976), is genetically controlled (i.e. a genotypic trait) and is not an artifact of physical changes that occur during maturation (desiccation) or isolation.

Structure of purified glutenin

A great deal of research has been carried out on the physicochemical properties of glutenin in an attempt to elucidate its structure in relation to functionality in breadmaking (for reviews see BIETZ *et al.*, 2973; BUSHUK, 1974; KASARDA *et al.*, 1976). This research has been beset with numerous difficulties because

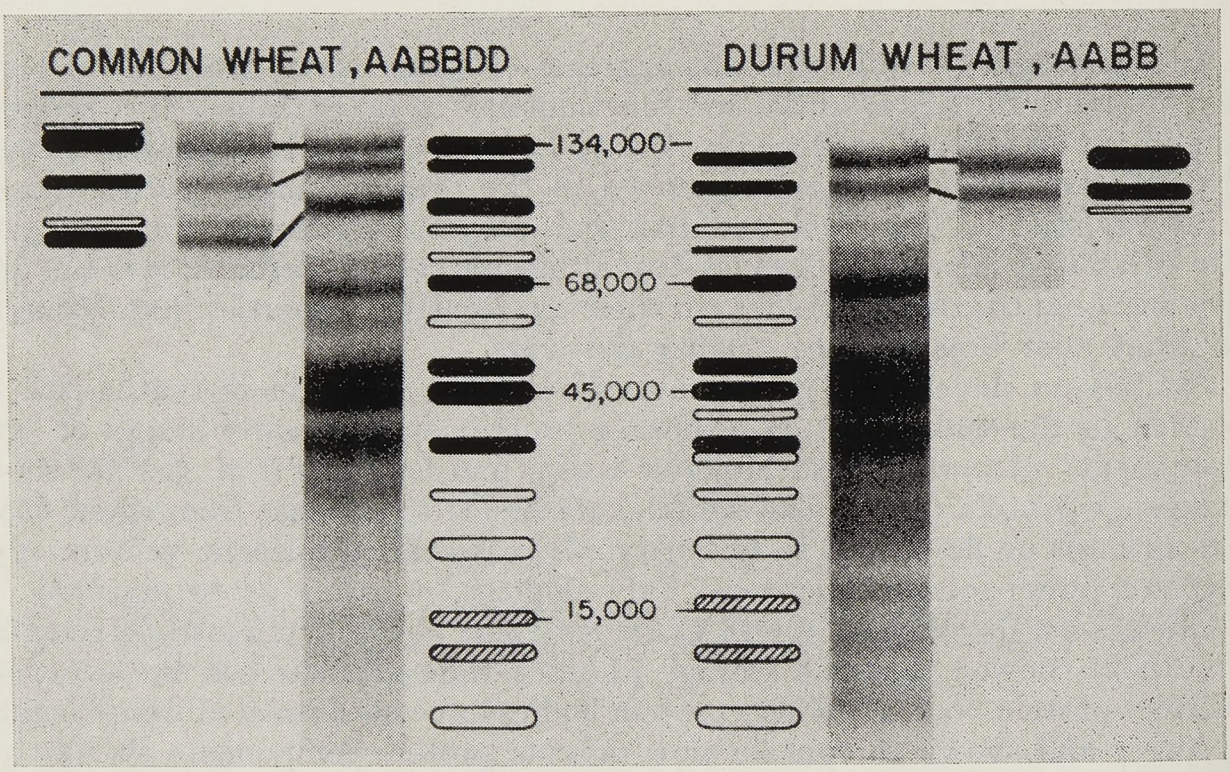

FIG. 2. — *SDS-PAGE of reduced bread and durum wheat glutenins.*

of the complex chemical and physical nature of glutenin. Some progress, however, has been made.

SDS-PAGE studies of reduced bread wheat glutenin (Fig. 2), has shown that this protein consists of approximately 17-20 polypeptide subunits (BIETZ and WALL, 1972; ORTH and BUSHUK, 1973*a*). The molecular weights of these subunits, determined by the same technique, range from 134 000 to 12 000 (KHAN and BUSHUK, 1976). The fact that glutenin must be treated with reducing

agents such as β-mercaptoethanol to release all of the subunits of glutenin has been interpreted to indicate that interpolypeptide disulfide bridges link the subunits into very large molecules with molecular weights in the millions (JONES *et al.*, 1961). However, recent information has cast doubts on the validity of the hypothesis that the functional glutenin comprises totally disulfide-crosslinked molecules. Much of the recent evidence supports the concept that the major structural feature of functional glutenin is the aggregation (association) by secondary forces such as hydrogen bonds and hydrophobic and ionic interactions (KASARDA *et al.*, 1976; KHAN and BUSHUK, 1978, 1979*a*; EWART, 1979). The details of the structure of the functional aggregates remain to be discovered.

The amino acid composition of glutenin (Table 1) shows that it contains many amino acids that can promote association of its subunits. It contains fairly

TABLE 1

Amino acid composition of glutenin (1, 2)

Lysine	12.5
Histidine	13
Arginine	20
Aspartic acid	23
Threonine	26
Serine	50
Glutamic acid	278
Proline	114
Glycine	78
Alanine	34
Cysteine	10
Valine	41
Methionine	12
Isoleucine	28
Leucine	57
Tyrosine	25
Phenylalanine	27
Tryptophan	8
Amide	240

(1) Amino acid residues per 100 000 g.
(2) KASARDA *et al.*, 1976.

high levels of hydrophobic amino acids such as leucine which, acting collectively, can promote strong protein-protein interactions. Low levels of basic and acidic amino acids coupled with high levels of glutamine (indicated by the high level of ammonia) can also promote protein-protein association. The chemical composition of glutenin and its subunits, therefore, possess the specific characteristics that can enhance protein-protein interactions which, in turn, would influence functionality in some of the processing steps in breadmaking.

The subunit composition of glutenin is affected by pH and ionic strength (KHAN and BUSHUK, 1977). Using tris-borate buffer of pH 8.9 and phosphate buffer of pH 7.3, KHAN and BUSHUK (1977) obtained cultivar differences in resolution on SDS-PAGE of certain subunits. This peculiar cultivar-dependent response of glutenin subunits to changes in pH or buffer ions may be relevant to

functional properties especially in a concentrated system such as dough. Minor differences in subunit composition (which obviously exist as shown by these results) could have a significant effect on the rheological properties of dough and thereby on bread quality.

Functional glutenin

In this section we shall present results that we consider relevant to the structure to function relationship of glutenin in breadmaking. Our results indicate that functional glutenin, that is, glutenin as it exists in gluten and dough, is a complex of covalently and non-covalently bound components. It is postulated

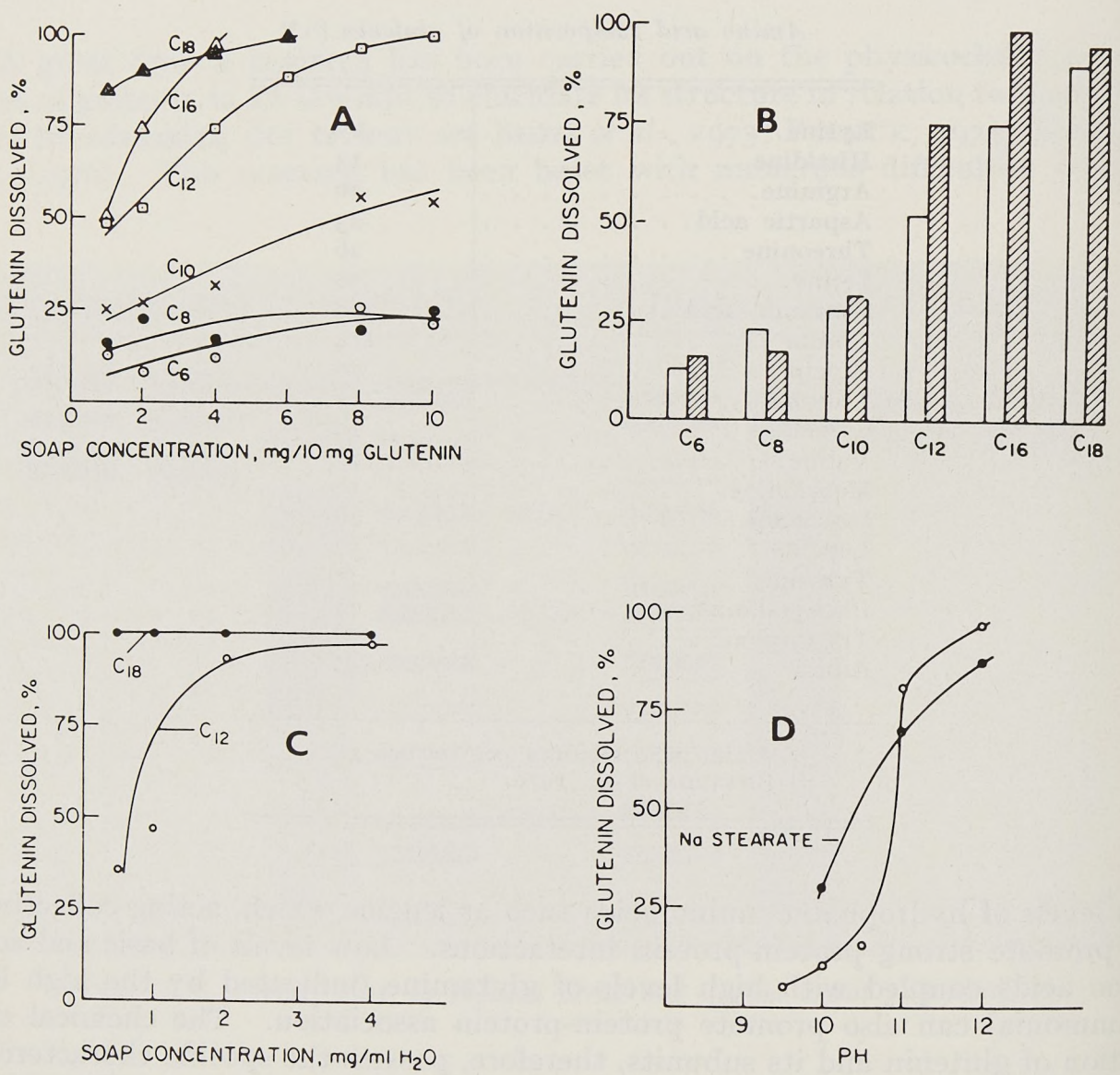

FIG. 3. — *Solubility of glutenin in soap solutions: A, solubility of glutenin in water containing increasing quantities of various soaps (10 mg glutenin per 2 ml water); B, relation between glutenin solubility and fatty acid chain length at two soap concentrations in water (2 mg and 4 mg (grid bars) soap per 10 mg glutenin in 2 ml of water); C, solubility of glutenin at varying dilutions of sodium dodecanoate (C_8) and sodium stearate (C_2) but at constant rates of soap: glutenin (4 mg soap per 5 mg glutenin in 1-8 ml water); and D, solubility of glutenin in solvents of different pH with and without (closed circles) 4 mg sodium stearate per 5 mg of glutenin in 1 ml of solvent.*

that the components, mainly proteins and trace quantities of carbohydrates and lipids, interact to varying degrees, depending on their quantities and their complexing capacity, to impart the characteristic visoelasticity and breadmaking quality to gluten and dough.

The key observation that indicates that glutenin is probably an aggregate of various components was made by KOBREHEL and BUSHUK (1977) when they successfully solubilized glutenin in sodium stearate-water mixtures. A detailed

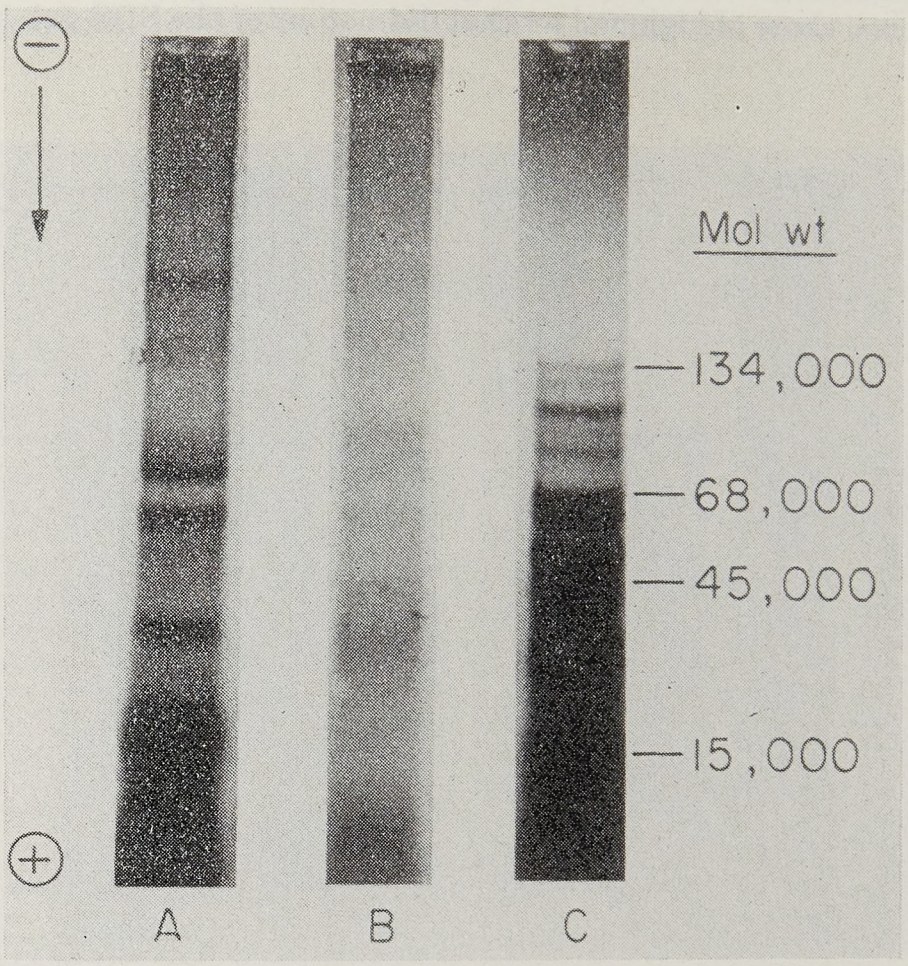

FIG. 4. — *SDS-PAGE of glutenin of the cultivar Red River 68: A, non-reduced acetic acid-soluble glutenin; B, non-reduced residue protein; and C, reduced acetic acid-soluble glutenin.*

study of the effect of soap concentration, fatty acid chain length, dilution and pH (Fig. 3A, B, C and D, respectively) showed that sodium stearate and sodium dodecanoate were the best soaps for solubilizing glutenin. Subsequent studies (KOBREHEL and BUSHUK, 1978) on the binding of soap to glutenin showed that there is an extremely strong interaction between glutenin and the soap. These results indicate that the insolubility of glutenin is largely due to extremely strong and specific hydrophobic interactions which can be destroyed by the amphiphilic soap molecule. We have therefore proposed that these strong hydrophobic interactions play a major role in the structure of functional (in breadmaking) glutenin (KHAN and BUSHUK, 1978, 1979*a*).

Other evidence that supports the aggregate hypothesis was obtained from our experiments on different fractionation and purification schemes (KHAN and BUSHUK, 1979*a*). A survey of the literature revealed that, in general, researchers took two main approaches to study structure-function relationships of glutenin.

The first group of researchers, those who used reconstitution and baking studies (SHOGREN *et al.*, 1969; ORTH *et al.*, 1972; Mac RITCHIE, 1979; BOOTH and MELVIN, 1979) to evaluate the influence of glutenin in breadmaking, used " crude " glutenin, that is, without extensive purification, from the Osborne solubility fractionation or similar procedures. The second group, those concerned with fundamental physiocochemical studies, used glutenin preparations that had been extensively purified by various procedures. To compare the two types of glutenin we have examined them in considerable detail by SDS-PAGE.

SDS-PAGE of non-reduced crude glutenin revealed that it comprises two types of proteins, those of high mol wt that did not enter the SDS-gel, but remained

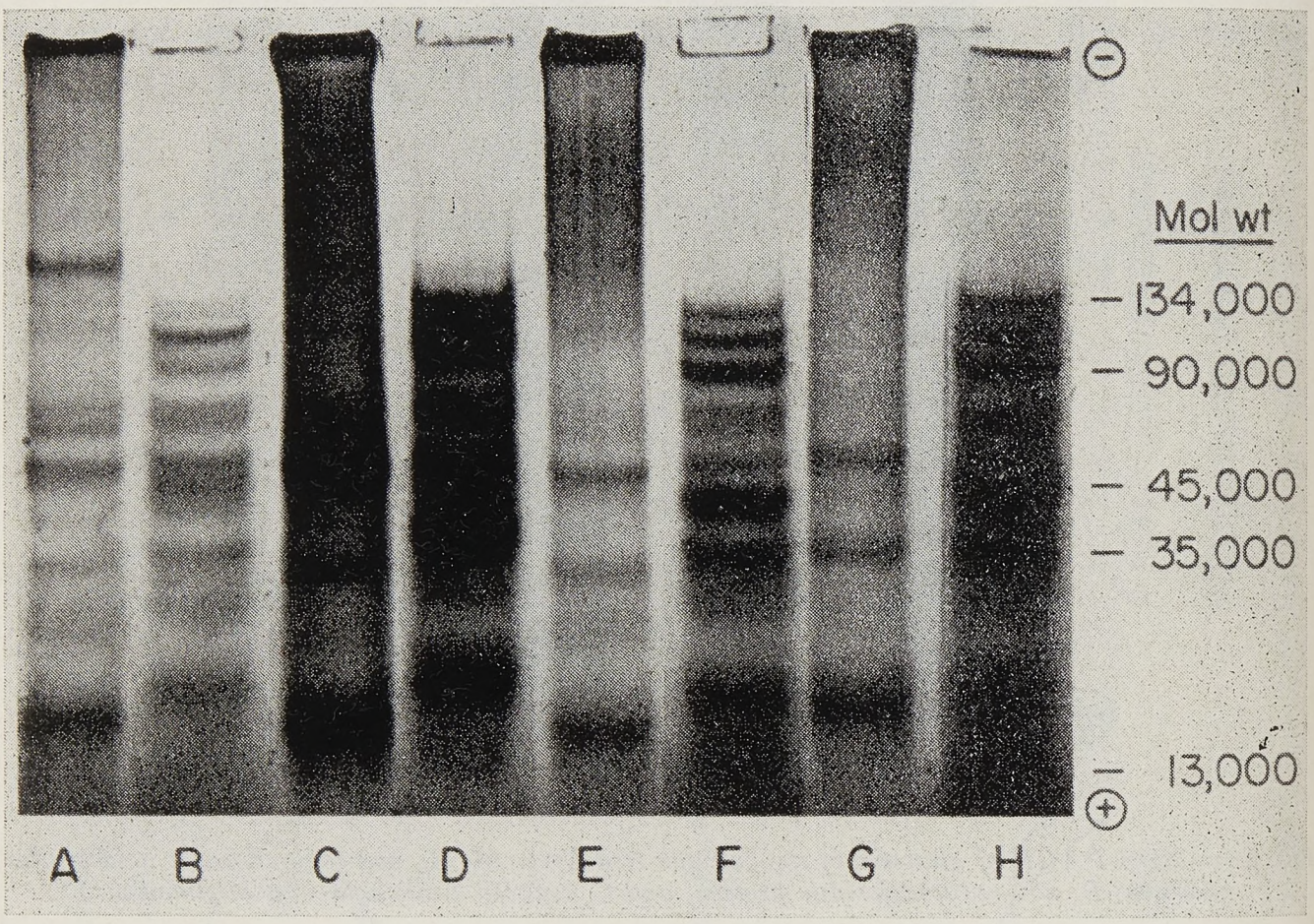

FIG. 5. — *SDS-PAGE of non-reduced and reduced acetic acid-soluble glutenin of different cultivars: A, D, E, G, non-reduced glutenin of Red River 68, Manitou, Ponca and Talbot, respectively; and B, D, F, H, reduced glutenin of Red River 68, Manitou, Ponca and Talbot, respectively.*

at the point of sample application, and those in the 68 000 to 12 000 mol wt region that entered the SDS gel (Fig. 4A). Comparison of the non-reduced soluble crude glutenin from different cultivars (Fig. 5) showed both qualitative and quantitative differences in the components that entered the gel. The components that entered the gel correspond, in mobility, to many of the subunits of reduced glutenin (patterns B, D, F, H). The SDS-PAGE pattern of the non-reduced residue protein (see Fig. 4B) also showed protein components that entered the gel. Therefore, both the soluble crude glutenin and the residue protein contain non-covalently bound low mol wt protein components in addition to the high mol wt protein.

Examination, by SDS-PAGE, of purified glutenin also showed the presence of non-covalently bound protein components that entered the SDS-gel. Figure 6B shows the pattern of highly purified unreduced glutenin (KHAN and BUSHUK, 1979*a*) prepared by the single-kernel procedure of BIETZ *et al.* (1975). It is seen that many prominent protein components are present in the SDS-gel in the 68 000 and lower mol wt region. These components correspond in mobility to many of the subunits of reduced total glutenin (Fig. 6A). Patterns C and D represent unreduced glutenin form the 3rd and 5th extracts with the SDS solvent using the single-kernel procedure (i.e. extensively purified glutenin). There are protein components, though faint, that enter the SDS gel from both extractions.

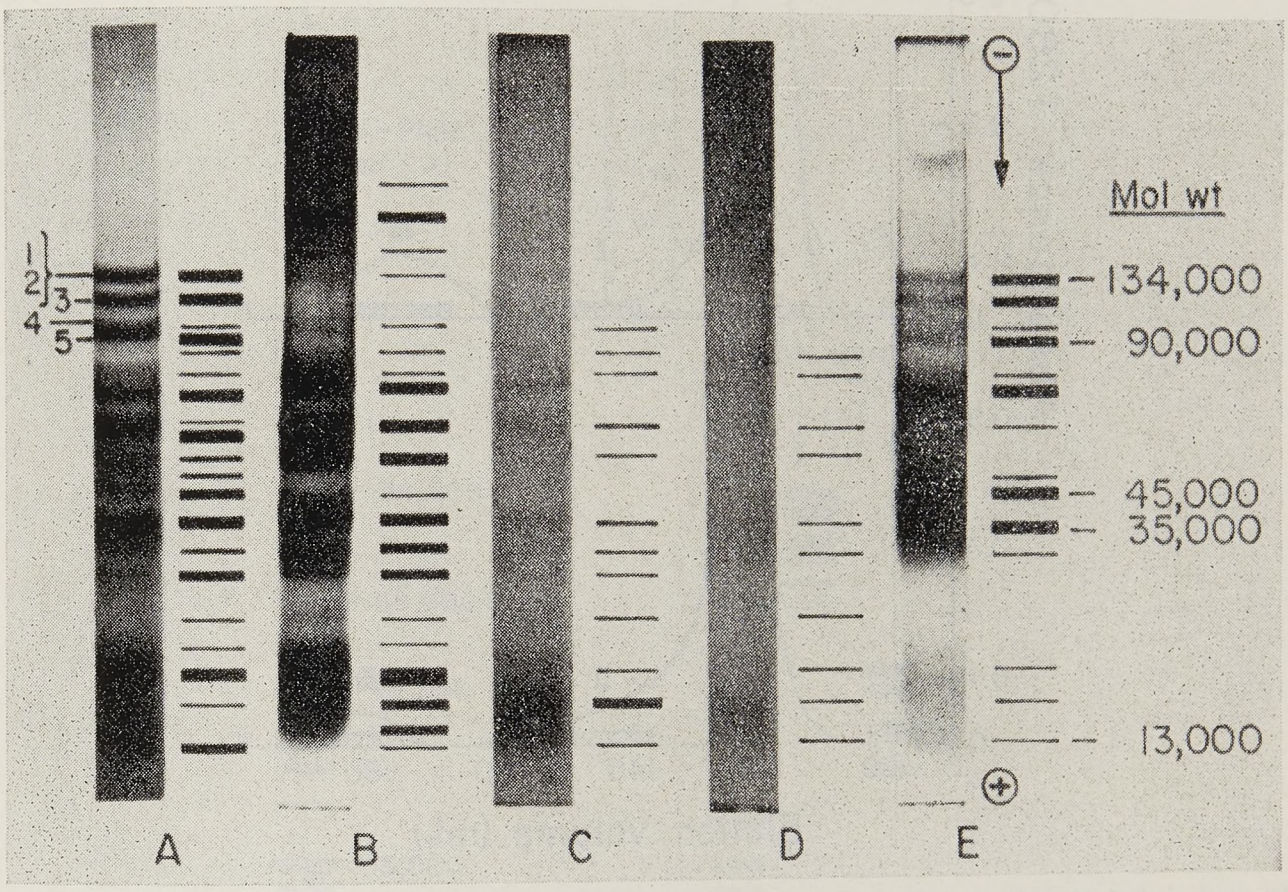

FIG. 6. — *SDS-PAGE of purified glutenin from the single-kernel procedure of* BIETZ *et al.* (1975) *of the cultivar Manitou: A, total reduced glutenin, B, C, D, first, third and fifth extracts with tris-borate-SDS buffer without reducing agent; and E, reduced glutenin from the residue after the fifth extraction.*

Accordingly, we have concluded that the components in the 68 000 and lower mol wt region, that appear in the pattern of reduced glutenin (see Fig. 6A), are bound in the functional glutenin complex by secondary forces.

There is good evidence that protein aggregates are present in the glutenin fraction isolated by gel-filtration chromatography. ORTH and BUSHUK (1973*b*) showed that the glutenin isolated by gel-filtration of an AUC extract of flour contained non-covalently bound protein components as indicated by PAGE at pH 3.2. DALEK-ZAWISTOWSKA *et al.* (1975) obtained an included protein peak by chromatography, on Sephadex G-200, of glutenin which was previously isolated by gel-filtration of gluten. HAMAUZU *et al.* (1979) subjected sodium stearate-

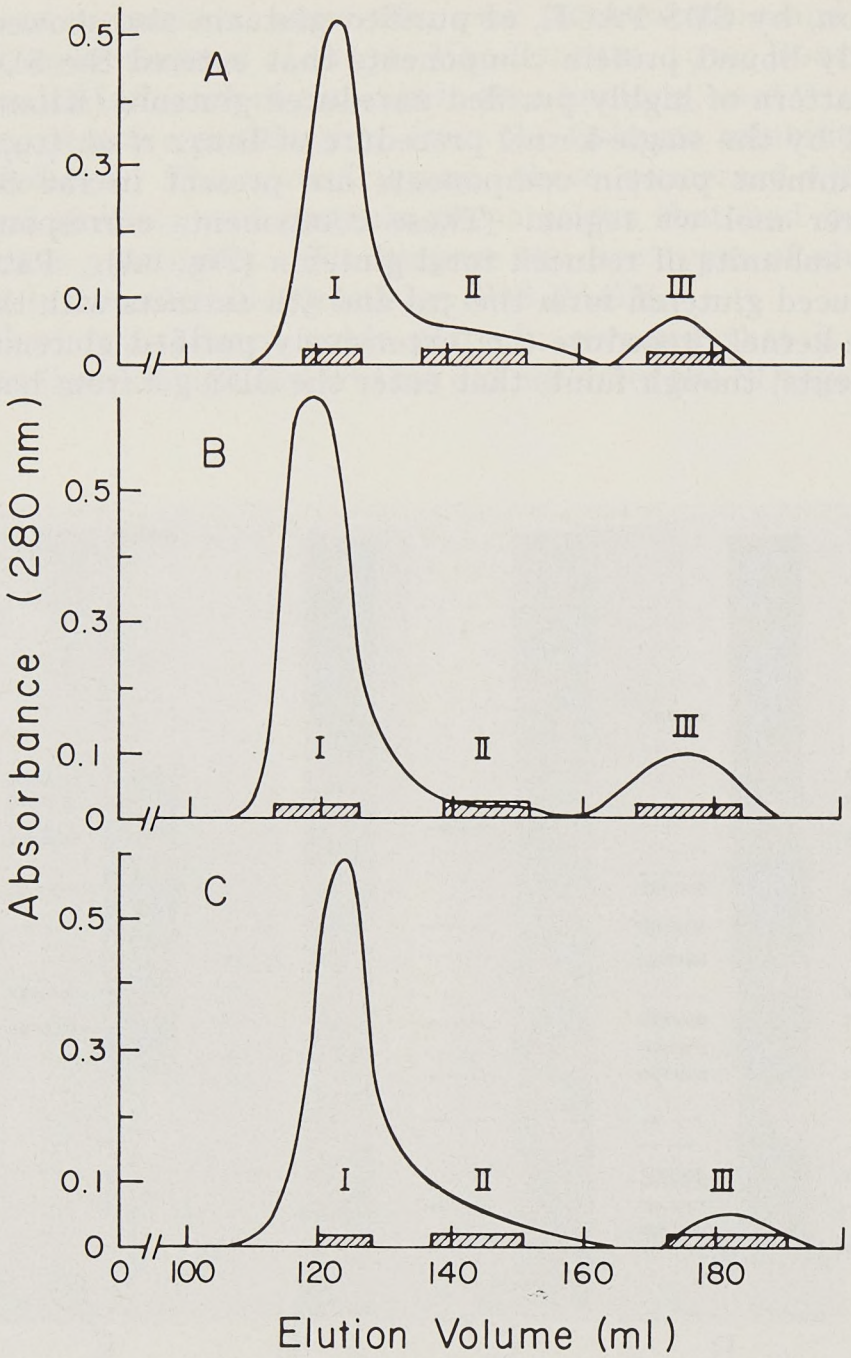

FIG. 7. — *Gel-filtration on Sephadex G-150 in SDS solvent of glutenin solubilized in: A, sodium stearate-water; B, Peak I (dialyzed and freeze-dried) from A solubilized in sodium stearate-water; and C, glutenin solubilized in tris-HCl-SDS. Eluant in all cases was 0.1 M tris-HCl-SDS (0.1 p. 100 w/v) buffer, pH 8.4. Peaks are indicated as I, II and III. Shaded areas indicate fractions that were pooled.*

solubilized purified glutenin, prepared by the ORTH and BUSHUK (1973*b*) method, to gel-filtration in SDS solvent and obtained included protein fractions (Fig. 7A) which contained low MW protein components (by SDS-PAGE). Peak I proteins (eluted in void volume) of Figure 7A were rechromatographed and an elution profile with included peaks (Fig. 7B) was again obtained. Gel-filtration of glutenin in tris-HCl-SDS solvent also produced included peaks (Fig. 7C). SDS-PAGE of the proteins of the included peaks showed components with MW in the 68 000 to 12 000 region. All of these observations support the hypothesis that glutenin contains low MW components held by secondary forces.

Gel-filtration of reduced and alkylated (RA-) glutenin has also demonstrated

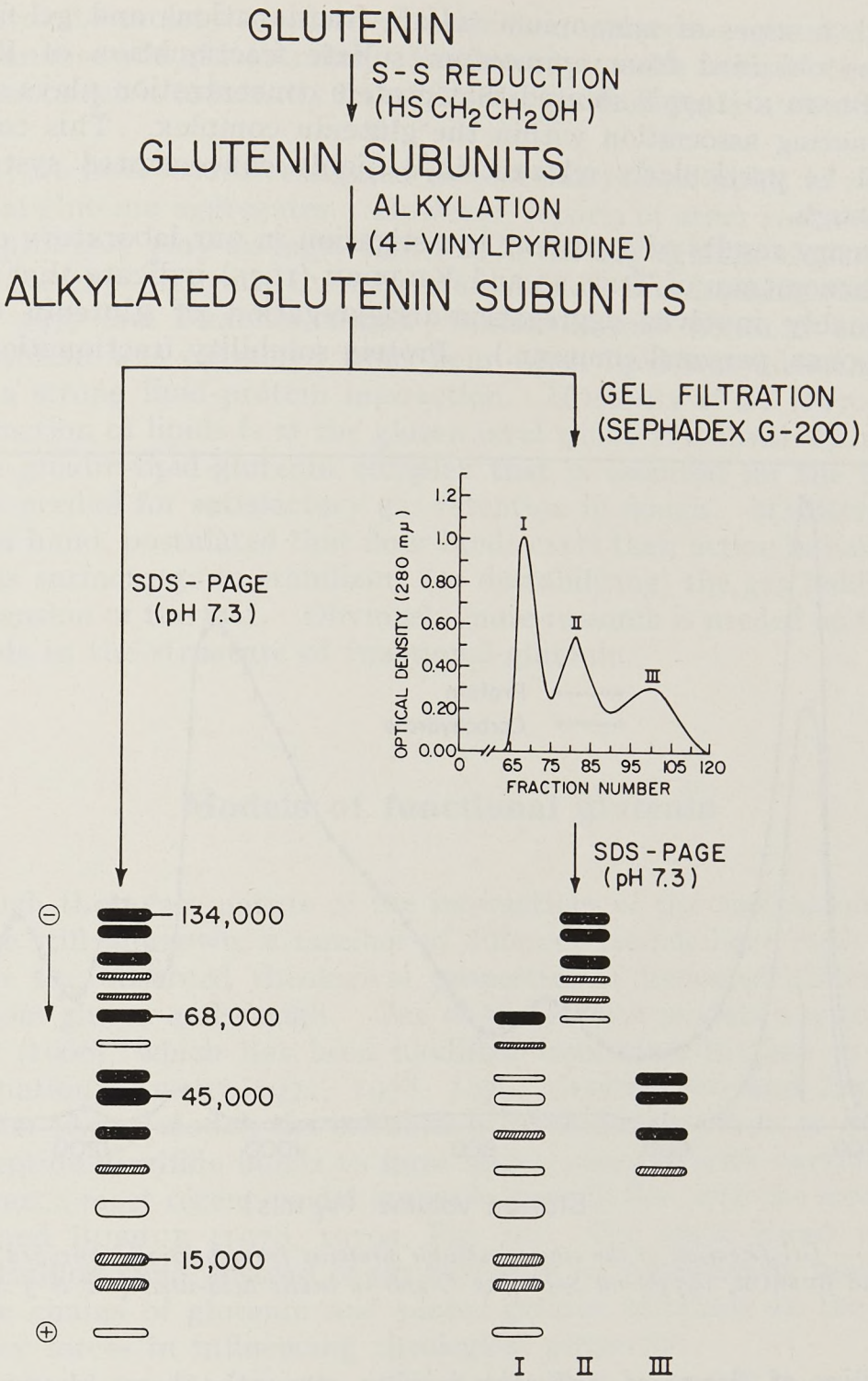

FIG. 8. — *SDS-PAGE of alkylated glutenin subunits of bread wheat (cv. Manitou) fractionated by gel-filtration on Sephadex G-200.*

that strong protein association exists in the glutenin complex (HUEBNER and WALL, 1974; ARAKAWA *et al.*, 1977; KHAN and BUSHUK, 1979*b*). Three peaks are generally obtained in gel-filtration of RA-glutenin (Fig. 8). However, peak I proteins are aggregates since they elute in the void volume on Sephadex G-200 (MW $<$ 200 000) but comprise protein components with MW of 68 000 and lower (Fig. 8). KHAN and BUSHUK (1979*b*) showed that most of peak I proteins were dissociated and eluted with 0.1 p. 100 SDS solvent at a volume on Sephadex G-200 consistent with their SDS-PAGE MW. It is concluded from these results that hydrophobic interactions play a major role in protein association in the glutenin complex.

Through a series of ammonium sulfate fractionations and gel-filtrations of the fractions obtained from ammonium sulfate fractionation of RA-glutenin, KHAN and BUSHUK (1979*b*) showed that protein concentration plays a significant role in promoting association within the glutenin complex. This concentration effect would be particularly relevant in a highly concentrated system such as gluten or dough.

Preliminary results of a current investigation in our laboratory of the " unmixing " phenomenon of TIPPLES and KILBORN (1975) indicate that this phenomenon probably involves aggregation-disaggregation of glutenin components (PAREDEZ-LOPEZ, personal commun.). Protein solubility fractionation and SDS-

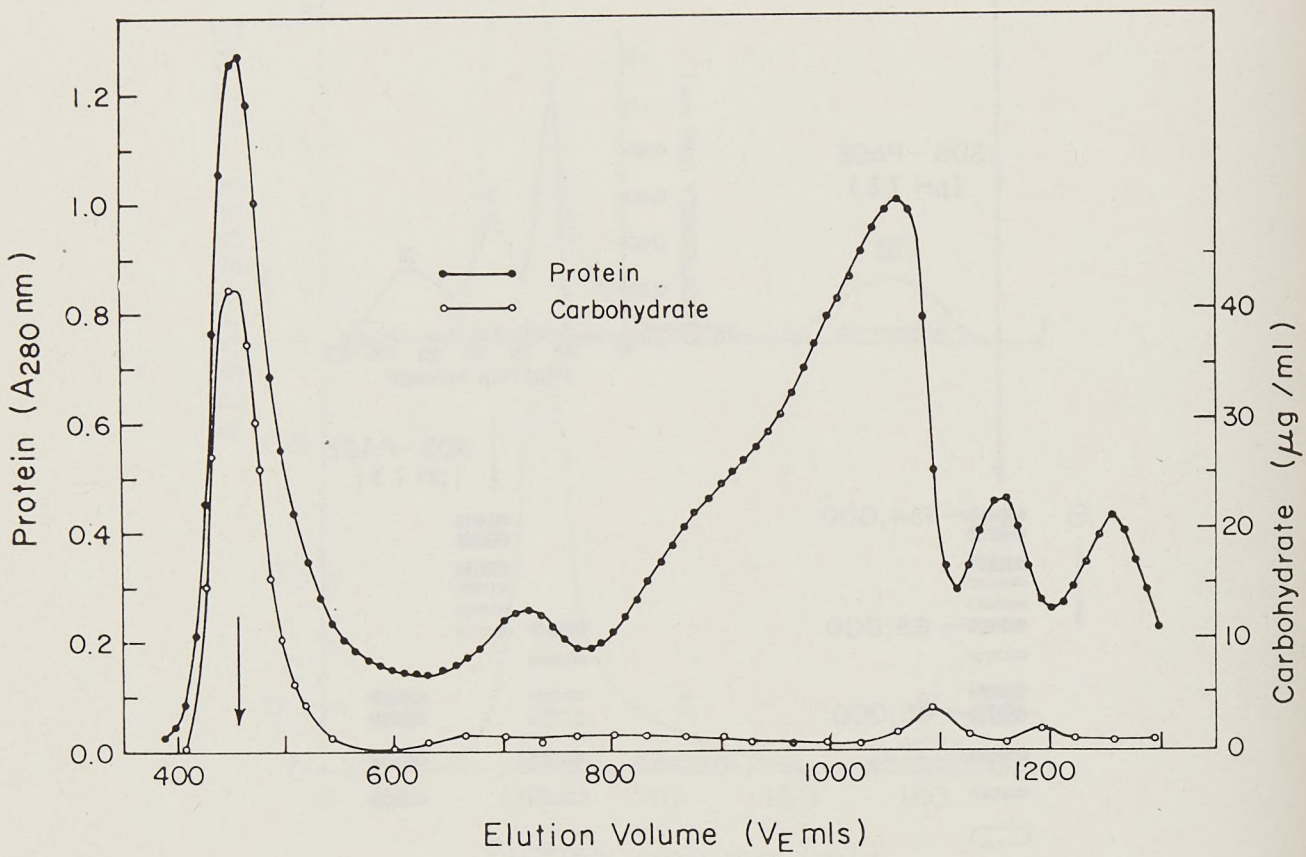

FIG. 9. — *Gel-filtration of the ethanol-soluble proteins from partially purified glutenin* (ORTH and BUSHUK, 1973*b*) *on Sephadex G-200 in acetic acid-urea (0.1 N-3 M) solvent.*

PAGE studies of flours of different mixing strengths have shown significant differences in the proportion of the acetic acid-soluble glutenin and its subunit composition in doughs prepared by various mixing treatments including unmixing.

The possible role of carbohydrate as an integral component of the functional glutenin complex is currently under investigation in our laboratory. In an earlier study (KHAN and BUSHUK, 1979*b*) we observed that a relatively significant amount of carbohydrate (ca 6 p. 100) was present in peak I proteins obtained from gel-filtration of RA-glutenin (Fig. 8). Subsequent investigations with the proteins in the ethanol supernatant from the purification (ORTH and BUSHUK, 1973*b*) of glutenin showed that a carbohydrate component(s) separates with high mol wt fraction which elutes in the void volume on Sephadex G-200 (Fig. 9). The SDS-PAGE of the reduced proteins of this high mol wt fraction shows a pattern similar to that of purified glutenin isolated by the pH precipitation method (ORTH and BUSHUK, 1973*b*). Sugar analysis (by paper chromatography) revealed that

the carbohydrate moiety consists of arabinose, galactose, glucose, xylose and traces of mannose and glucosamine. Preliminary results indicate that the carbohydrate component is covalently linked to the glutenin protein through hydroxyproline.

As yet, we have not investigated the specific role of lipids in the formation of functional glutenin aggregates. However, reports of other researchers indicate that lipids probably play an important role in the aggregation of glutenin components and thereby influence their functionality in breadmaking (for reviews see MORRISON, 1977 and FRAZIER, 1979). SIMMONDS and WRIGLEY (1972) showed that less protein was extracted from flour when lipid was added to the flour, indicating a strong lipid-protein interaction. HOSENEY *et al.* (1970), suggested that the function of lipids is at the gluten level where it is involved in the formation of the gliadin-lipid-glutenin complex that is essential for the formation of membranes needed for satisfactory gas retention in dough. MACRITCHIE (1977), on the other hand, postulated that flour lipids exert their action in baking through their role as surfactants in stabilizing (or destabilizing) the gas bubble structure during expansion of the loaf. Obviously more research is needed on the potential role of lipids in the structure of functional glutenin.

Models of functional glutenin

Although the exact nature of the interactions of the various components of glutenin are still unknown, a number of different models have been proposed to account for the observed rheological properties of hydrated glutenin and, by extrapolation, gluten and dough. One of the earliest models was that proposed by EWART (1968), which has been modified from time to time to incorporate new information (EWART 1972, 1978, 1979; GREENWOOD and EWART, 1975). In the earlier Ewart model, the subunits of glutenin are joined to each other by interpolypeptide disulfide bonds to form large molecules with various concatenations. Ewart's most recent model (1979) is more in line with the model proposed by KHAN and BUSHUK (1978, 1979*a*, Fig. 10). The latest Ewart model shows only one disulfide bond, instead of the two as in his penultimate model, between polypeptide chains of glutenin and places greater emphasis on the importance of secondary forces in influencing rheological properties.

KASARDA *et al.* (1976), on the other hand, have proposed a model in which there are no interpolypeptide disulfide bonds in glutenin; all of the disulfides are intrapolypeptide type. They postulated that the intrapolypeptide disulfide bonds constrain the glutenin subunits into specific conformations which facilitate the interaction of specific secondary forces between adjacent polypeptides thereby forming the functional aggregate. Furthermore, they postulated that these secondary forces are so strong that they cannot be disrupted by any known solvent unless the disulfide bonds are first cleaved (as by reducing agents). Reduction of the intrapolypeptide disulfide bonds destroys the subunit conformation, which, in turn leads to disruption of the specific interpolypeptide secondary forces thereby releasing the glutenin subunits.

Our model of glutenin contains both interpolypeptide (and intrapolypeptide) disulfide bonds and secondary forces holding the subunits together in the functional glutenin (see Fig. 10). Our results indicate that the subunits of MW 68 000 and

lower, glutenin (Glu) I, are held in the complex through secondary forces (hydrogen bonds, ionic and hydrophobic interactions) through protein-protein interactions and protein-intermediate (carbohydrate, lipid) — protein interactions. These subunits provide a measure of viscous flow in the model. Glu II, on the other hand, are those glutenin subunits with MW above 68 000. These subunits are jointed to each other through at least one interpolypeptide disulfide bond. Glu II contributes the characteristic elasticity to glutenin. Glu I subunits are linked to Glu II by secondary forces. This model can, in a qualitative way, account

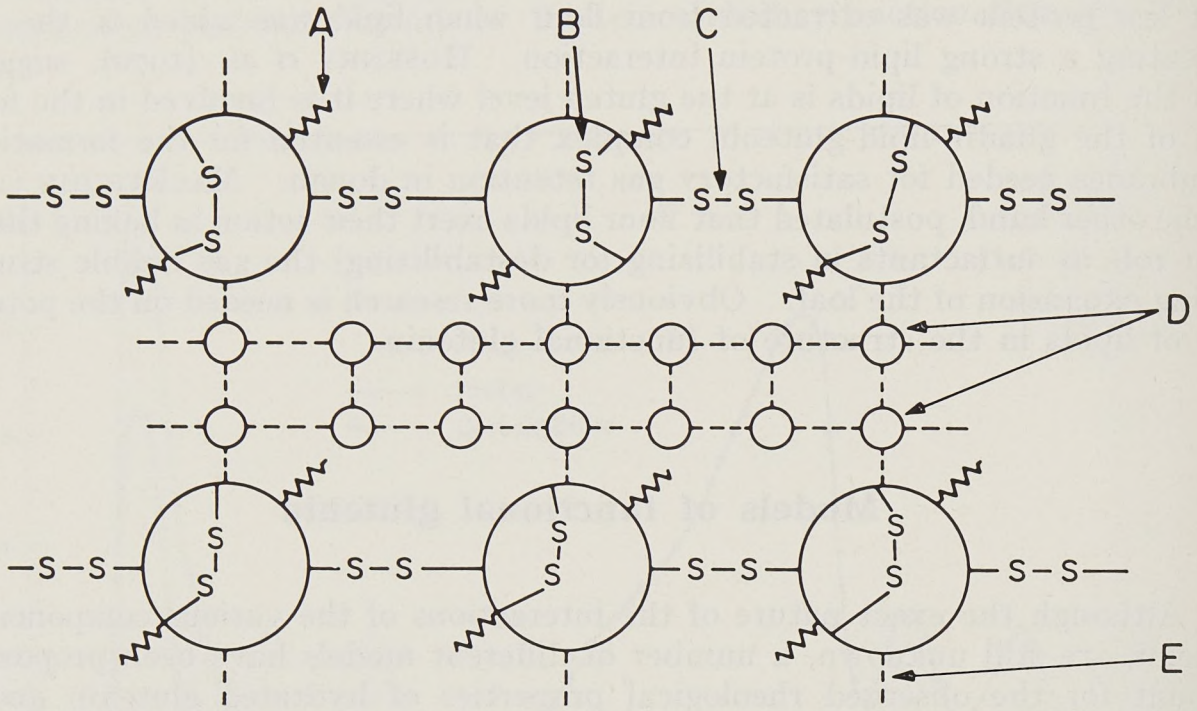

FIG. 10. — *Schematic representation of the model of functional glutenin: A, glutenin II subunits; B, intrapolypeptide disulfide bond; C, interpolypeptide disulfide bond; D, glutenin I subunits; and E, secondary bonds, e.g., hydrogen bonds and hydrophobic interactions.*

for most of the observed rheological properties of gluten and dough. Further refinement of the glutenin structure and the model of functional glutenin remains for future research.

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Résumé

La gluténine fonctionnelle : complexe des constituants liés de façon covalente et non-covalente

La gluténine est la protéine de la farine de blé qui reste dans le résidu insoluble après extractions successives par les solutions de chlorure de sodium 0,5 N et d'éthanol 70 p. 100. Elle représente 35 à 40 p. 100 de la protéine de la farine et contribue à l'élasticité des pâtes et du gluten humide. Elle contribue beaucoup à la qualité boulangère et elle est responsable de la plupart des différences génotypiques de la valeur boulangère entre cultivars de la même classe de blé.

L'utilisation de l'électrophorèse en gel de polyacrylamide en présence de dodecylsulfate de sodium (SDS PAGE) a fait considérablement progressé la connaissance de la structure de la gluténine en relation avec ses propriétés fonctionnelles. Les résultats obtenus par SDS-PAGE montrent que la gluténine réduite comporte environ 17 sous-unités, possédant du poids moléculaire compris entre 1 200 et 134 000.

Des études de solubilité dans des solvants amphiphiles (p. ex stearate de sodium) et des solvants dissociants (par exemple mélange acide acétique — urée — bromure de bromure de cetyltrimethyl ammonium) montrent que les interactions hydrophobes jouent un rôle important dans l'insolubilité et la stabilité rhéologique du complexe fonctionnel de la gluténine.

Tandis que quelques unes des sous-unités peuvent être libérées (c'est-à-dire solubilisées) du complexe à l'aide de réactifs amphiphiles, la dislocation complète en sous-unités demande la réduction des liaisons disulfides.

Une étroite association a été démontrée entre un glucide et quelques sous-unités à poids moléculaire élevé de la gluténine. Des résultats préliminaires indiquent que la partie glucidique est liée de façon covalente à la protéine.

La glycoprotéine semble être impliquée dans l'agrégation de sous-unités gluténiques spécifiques en complexes plus importants. Des analyses qualitatives des constituants des sucres de la partie glucidique indiquent la présence de pentoses et d'hexoses. Ces études nous ont conduit à un nouveau modèle de la gluténine fonctionnelle dans lequel la plupart des sous-unités sont maintenues ensemble, et au sein d'une matrice réticulée par ponts disulfurés, par des interactions secondaires (interactions hydrophobes, ioniques, liaisons hydrogènes). La matrice qui contient principalement les sous-unités à poids moléculaire élevé, joue un rôle primordial dans la fonctionalité du complexe de la gluténine en panification.

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Properties of wheat gel proteins

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Summary

Wheat gel proteins, the amount of which is related to the baking strength of wheat, are those proteins which occur in a highly hydrated insoluble residue remaining after flour extraction with water and 2-chloroethanol. They were fractionated into water soluble gel and water insoluble gel by dialysis against water. Water soluble gel was separated into only two fractions by gel filtration either on Bio Gel P 60 or Sephacryl S 200. Both peaks included the lowest and the medium molecular weight subunits, while the high molecular weight subunits only occured in the first peak. By solubilizing water soluble gel in 0 to 0.1 M NaCl solution, it was shown that the High molecular weight subunits were the first insolubilized when the NaCl molarity increase. A large part of the Medium Molecular Weight subunits was still solubilized at the same ionic strength. Water insoluble gel was fractionated according to solubility by two different extraction procedures. Further studies of solubilization with sodium salt acids suggests that the bonds involved into the association of subunits to constitute native gel proteins are mainly no covalent types.

Key words : *wheat — gel protein — solubility — fractionation.*

For many years the study of the factors responsible for bread-making quality have been concentrated on the gluten proteins. Major emphasis has recently been placed upon the acetic acid insoluble glutenin and gel protein fraction. MECHAM and coworkers (1972) used the term gel protein to refer to proteins that occured in the highly hydrated insoluble material remaining after exhaustive extraction of flour with dilute acetic acid. For MEREDITH (1961), a gel protein was obtained after extraction of gluten with formic acid. According to JENNINGS (1978) a gel was formed when flour was suspensed in a phenol-acetic acid water solvent.

In our laboratory gel protein was formed in 2-chloroethanol. We have shown that there is a good correlation between the baking quality score and the gel protein content of flour (FEILLET *et al.*, 1976; JEANJEAN et FEILLET, 1979). The higher the thickness of gel protein, the better would be the baking quality of the variety. Gel protein content ranged from 5-6 mm for non-breadmaking wheat as " Clement " and " Maris Huntsman " to 15 mm in superior wheat varieties

such as " Florence Aurore " and " Rex " (Fig. 1). Gel protein content is mainly a varietal characteristic little influenced by growing conditions of the wheat plant. Determination of gel protein content could be utilized as a rapid and simple biochemical test which allows prediction of the breadmaking quality of wheat.

The nature of the gel protein fraction is not well know. It was stated by COLE *et al.* (1973) that gel protein is a fraction of glutenin, whereas according to BIETZ and WALL (1975) it consists both of glutenin and low molecular weight gliadin — like components. In a earlier work carried out to compare the effects of isolation conditions on physicochemical properties of gel protein we have shown that gel protein and glutenin were very similar if not identical according to their amino acid composition and molecular weight subunit distribution. They differed only by their electrophoretic pattern in aluminium lactate buffer

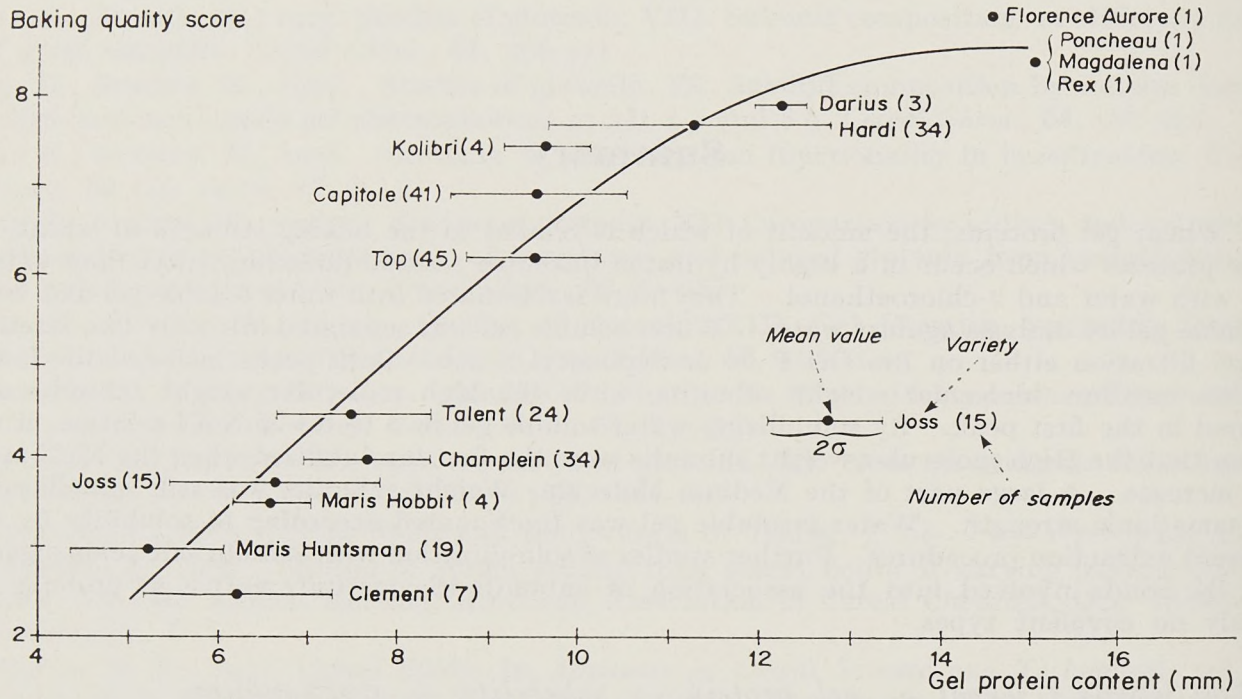


FIG. 1. — *Relation between the gel protein content (mm) and the baking quality score of wheat varieties*

(JEANJEAN and FEILLET, 1978). It was postulated that the main differences between gel protein and glutenin were the strength and the nature of the associations between essentially the same protein components.

The present work is a development of this earlier study and was undertaken to obtain more information on the structure and the properties of that gel protein complex and especially the high molecular weight subunits specific for glutenin. In this investigation, both the water soluble and water insoluble gel proteins were studied.

Materials and methods

Extraction of gel proteins

Gel proteins were extracted from a commercial untreated wheat flour (*Triticum Vulgare*) (protein content, 10.5 p. 100 d.b.) according to the method of JEANJEAN and FEILLET (1979). Flour was extracted twice by stirring for 15 min.

with distilled water and centrifuged 15 min. at 38 000 x g. Supernatants were discarded and insoluble material extracted with 70 p. 100 2-chloethanol (CE), pH = 1.8 (70 : 30, CE/Water, V/V) by stirring for 15 min. The gel layer was then recovered from the upper layer of the centrifuged sediment (Fig. 2).

After extraction gel proteins were dialyzed against deionized water during 48 hours, then fractionated by centrifugation (30 min. at 38 000 x g). The supernatant was called water soluble gel protein (WSG) and the residue was called water insoluble gel protein (WIG). Both were freeze-dried.

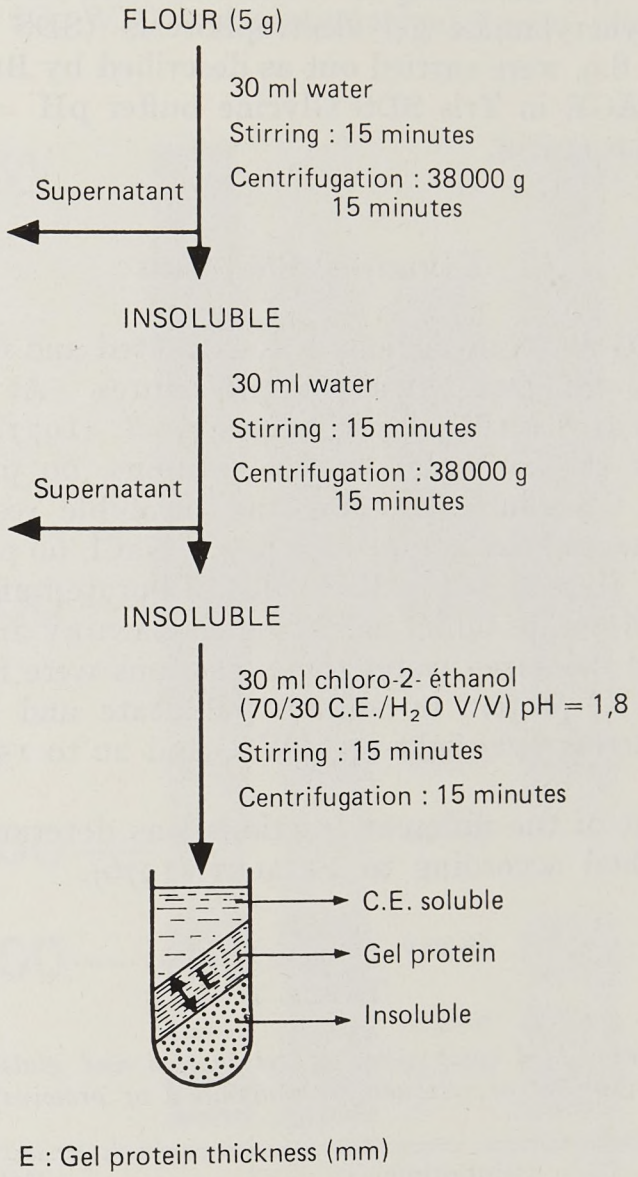


FIG. 2. — *Determination of gel protein content*

Gel filtration

Bio Gel P 60 (Bio-Rad laboratories) and Sephacryl S 200 (Pharmacia) were used for gel filtration of WSG protein. Bio Gel P 60 (molecular weight exclusion limit of 60 000) was equilibrated in distilled water and poured into a glass column (0.9 × 30 cm). Flow rate was of 6 ml/hour. Sephacryl S 200 (Molecular weight exclusion limit of 250 000) was equilibrated in 70 p. 100 CE (pH = 6.0) and packed into a glass column (0.9 × 40 cm). Flow rate was of 10 ml per hour.

WSG protein samples were dissolved either in water or in 70 p. 100 CE to give solutions containing 1 p. 100 protein by weight. Column effluents were collected in 2 ml fractions and absorbance was measured at 280 mm. The desired fractions were combined, dialyzed against deionized water and freeze-dried before electrophoresis.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was carried out in aluminium lactate buffer pH = 3.2 according to KOBREHEL and FEILLET (1971). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) in Tris SDS Borate buffer pH = 8.9, were carried out as described by BIETZ and WALL (1972) and vertical SDS PAGE in Tris SDS Glycine buffer pH = 8.3 as described by PAYNE and CORFIELD (1979).

Extraction Procedures

WIG (1 g of WIG in 50 ml solvent) was extracted and fractionated according to solubility by two different extraction procedures. At first, by a modified OSBORNE procedure as described by FEILLET *et al.* (1977). This fractionation gives four solubility classes: water soluble proteins, 60 p. 100 ethanol-soluble proteins, 70 p. 100 CE-soluble proteins and insoluble residue proteins. In a second time, by sequential extraction with 0.5 M NaCl, 60 p. 100 ethanol, 0.025M borate buffer (pH = 10), 0.5 p. 100 SDS-0.025 M borate buffer and 1. p. 100 mercaptoethanol-0.025 M borate buffer according to LANDRY and MOUREAUX (1970). After dialysis against deionized water these fractions were freeze-dried.

Solubility of WIG protein in sodium palmitate and sodium stearate was also examined by stirring overnight 1 g of WIG and 20 to 150 mg of sodium salts in 25 ml water.

Nitrogen content of the different fractions was determined by a semi automatic Kjeldahl method according to FEILLET (1976).

TABLE 1

Amino acid composition of gel protein and glutenin (number of residues per 100 000 g of proteins)

Amino acid	Glutenin(a)		Gel protein(b)		
	Gel Filtration	pH Precipitation	Total	Water Soluble	Water Insoluble
Lysine	13	19	12	7	19
Arginine	21	26	23	18	28
Glutamic acid	305	259	282	320	259
Aspartic acid	23	38	34	22	39
Proline	110	107	126	140	100
Glycine	63	79	70	49	80
Phenylalanine	38	29	31	36	34

(a) ORTH and BUSHUK (1973).
(b) JEANJEAN and FEILLET (1978).

Results and discussion

After dialysis against deionized water, the gel protein have been separated by centrifugation in a WSG and a WIG. Comparison of the amino acid composition (Table 1) of WSG and WIG to that of glutenin indicated that amino acid composition of the WIG was almost similar if not identical to that of the pH-glutenin. Whereas WSG had slightly more glutamic acid and proline and less glycine and lysine than glutenin.

SDS-PAGE of WSG, WIG and glutenin had a very close subunit composi-

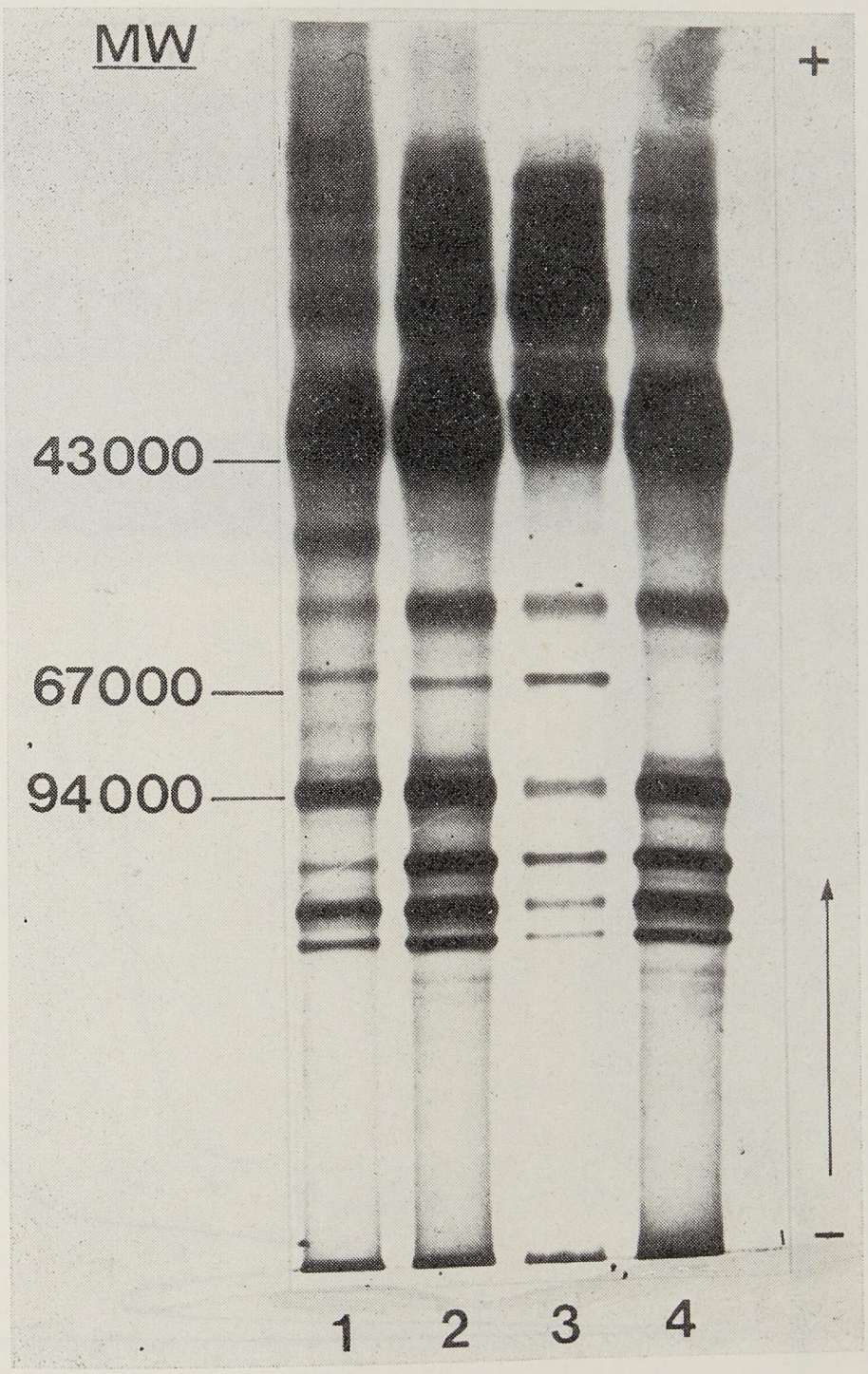


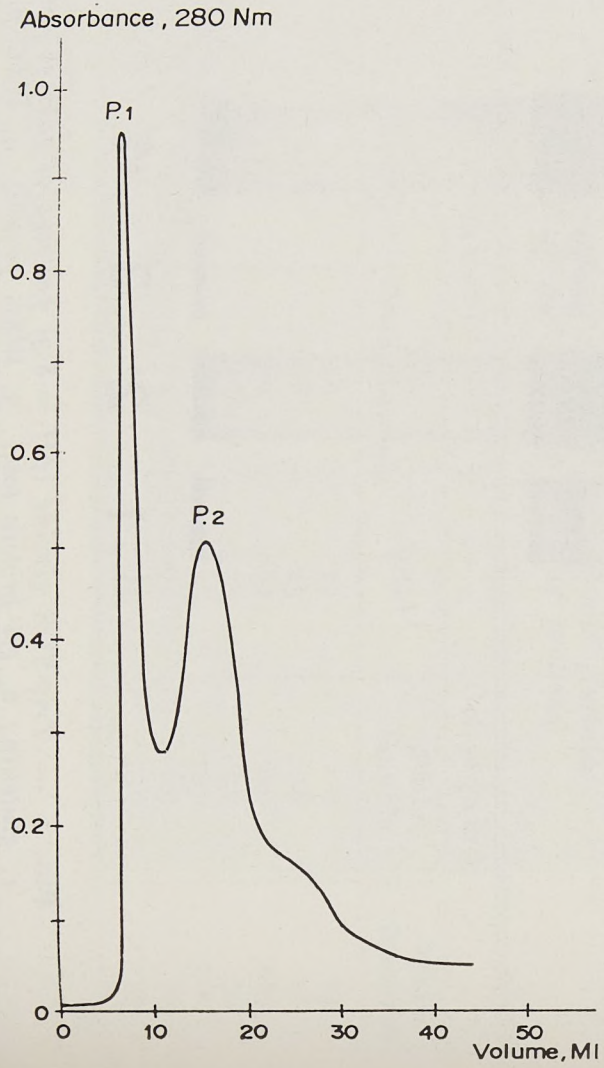
FIG. 3. — *Tris-SDS glycine (pH = 8.3) patterns of reduced samples: 1, glutenin; 2, gel protein total; 3, WSG protein; 4, WIG protein*

FIG. 4.

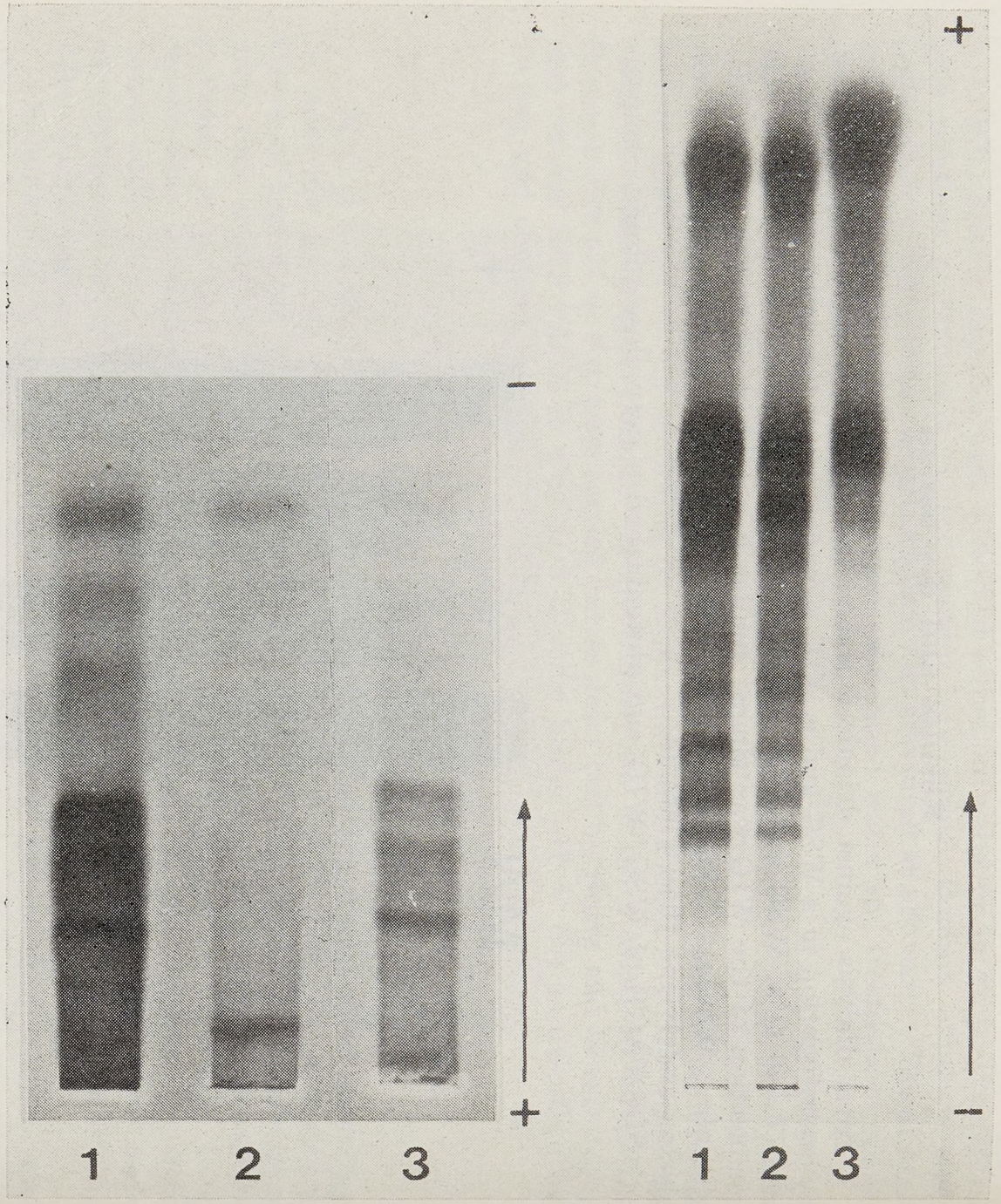
4a. — Bio Gel P 60 elution profile of WSG. Column : 0.9 × 30 cm ; solvent : water ; flow rate : 10 ml/hour ; 10 mg sample.

4b. — Aluminium lactate (pH = 3.2) PAGE patterns of 1, WSG ; 2, peak 1 ; 3, peak 2.

4c. — Tris SDS borate (pH = 8.9) PAGE patterns of reduced samples: 1, WSG ; 2, peak 1 ; 3, peak 2.



a



b *c*

tion (Fig. 3). In the WIG, subunit of molecular weight about 65 000 was missing. The presence in the WSG of the high molecular weight subunits specific for glutenin should be underlined.

Water soluble gel protein

The WSG protein was fractionated by gel filtration and by NaCl insolubilization. Gel filtration studies were carried out without the use of reducing agents in order to obtain information on the native components of the WSG. One of the major problems encountered in the fractionation of the WSG protein, was its insolubility in salt solutions. Therefore filtration was made using either water or 2-chloroethanol.

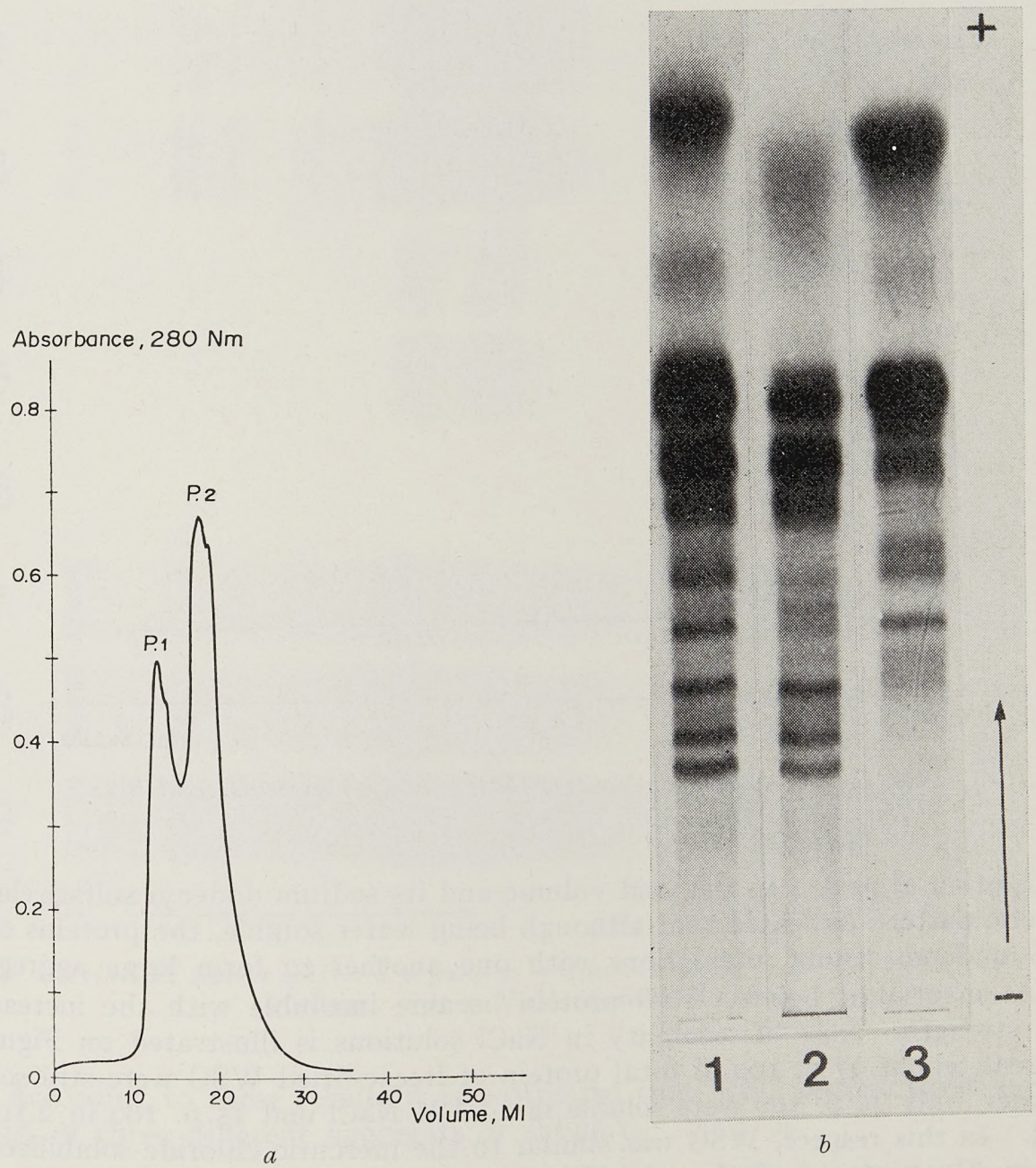


FIG. 5.

5*a*. — *Sephacryl S 200 elution profile of WSG. Column : 0.9 × 40 cm ; solvent : 70 p. 100. 2 C.E. ; flow rate : 6 ml/hour ; 10-mg sample.*

5*b*. — *Tris SDS borate (pH = 8.9) PAGE patterns of reduced samples : 1, WSG ; 2, peak 1 ; 3, peak 2.*

Figure 4*a* shows the elution profile obtain on Bio Gel P 60. The WSG was fractionated into two peaks, the first peak was eluted in the void volume. In aluminium lactate electrophoresis (Fig. 4*b*), peak 1 pattern consisted of a single slow moving component and a fast moving component. There were also some proteins remaining in the starting slot. Peak 2 was more heterogeneous. After reduction with mercaptoethanol (Fig. 4*c*), SDS-PAGE pattern of peak 1 was similar to that of the reduced WSG protein. In peak 2, the high molecular weight subunits were missing.

By gel filtration on Sephacryl S 200 two peaks were also obtained (Fig. 5*a*). Except the absence of the fast moving component, the SDS-PAGE pattern of peak 1 was close to that of the WSG. The highest molecular weight subunits were missing in peak 2 (Fig. 5*b*).

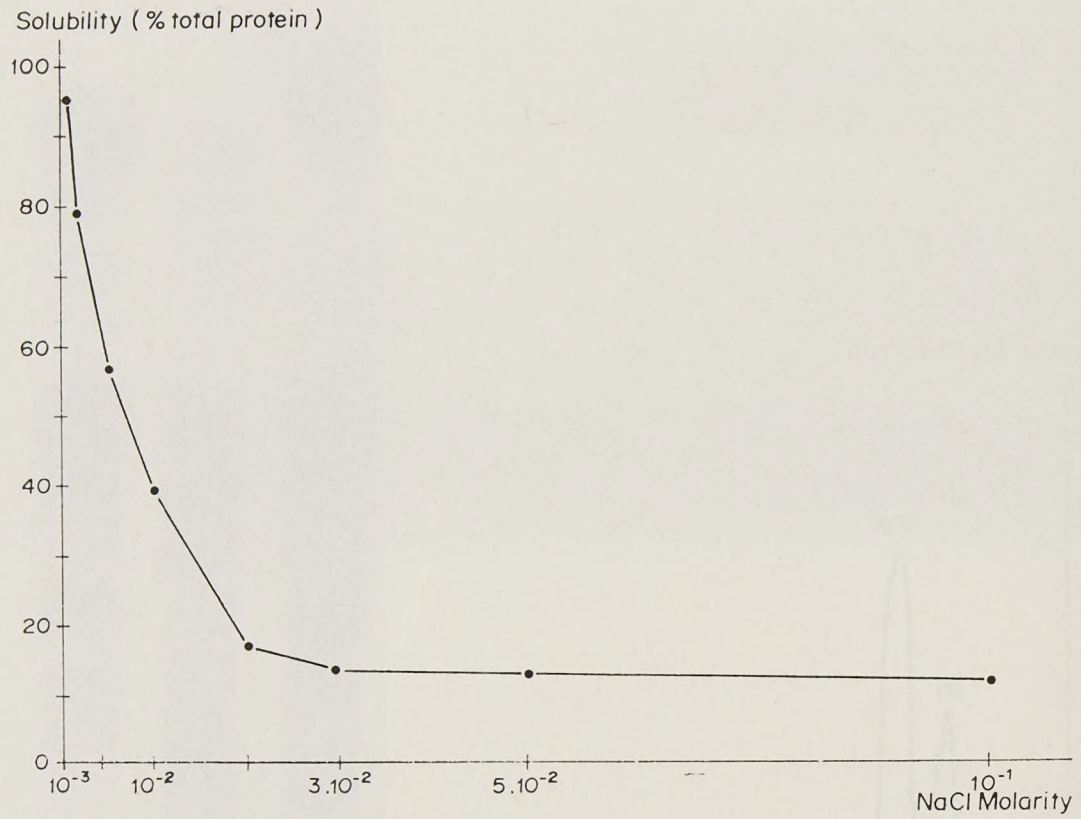


FIG. 6. — *Influence of NaCl molarity on WSG proteins solubility.*

Elution of peak 1 in the void volume and its sodium dodecyl sulfate electrophoretic pattern indicated that although being water soluble, the proteins of the WSG undergo strong interactions with one another to form large aggregates.

As mentioned before, WSG protein became insoluble with the increase in ionic strength. Loss of solubility in NaCl solutions is illustrated on Figure 6. Whereas, about 97 p. 100 of total protein of freeze-dried WSG were still soluble in water, only 40 p. 100 were soluble in 10^{-2} M NaCl and 15 p. 100 in 2.10^{-2} M NaCl. In this respect, WSG was similar to the mercuric chloride-solubilized gel protein (COLE *et al.*, 1976) and glutenin (HUEBNER, 1970) that were also salt sensitive.

We observe large variations in the SDS-PAGE pattern of NaCl soluble fractions (Fig. 7). For the low NaCl molarity only some minor difference were visible between the pattern of NaCl soluble fractions and WSG. In the 10^{-3} and the 10^{-2}

M soluble fractions, the high molecular wegiht subunits were missing. For the higher NaCl concentrations, only the low molecular weight subunits were present. So, it seems that the high molecular weight subunits were the first to be insolubilized followed by the medium molecular weight subunits. But if we examine the NaCl insoluble corresponding fractions (Fig. 7), even at low NaCl molarity, medium molecular weight subunits and traces of low molecular weight subunits were also present in the electrophoretic patterns.

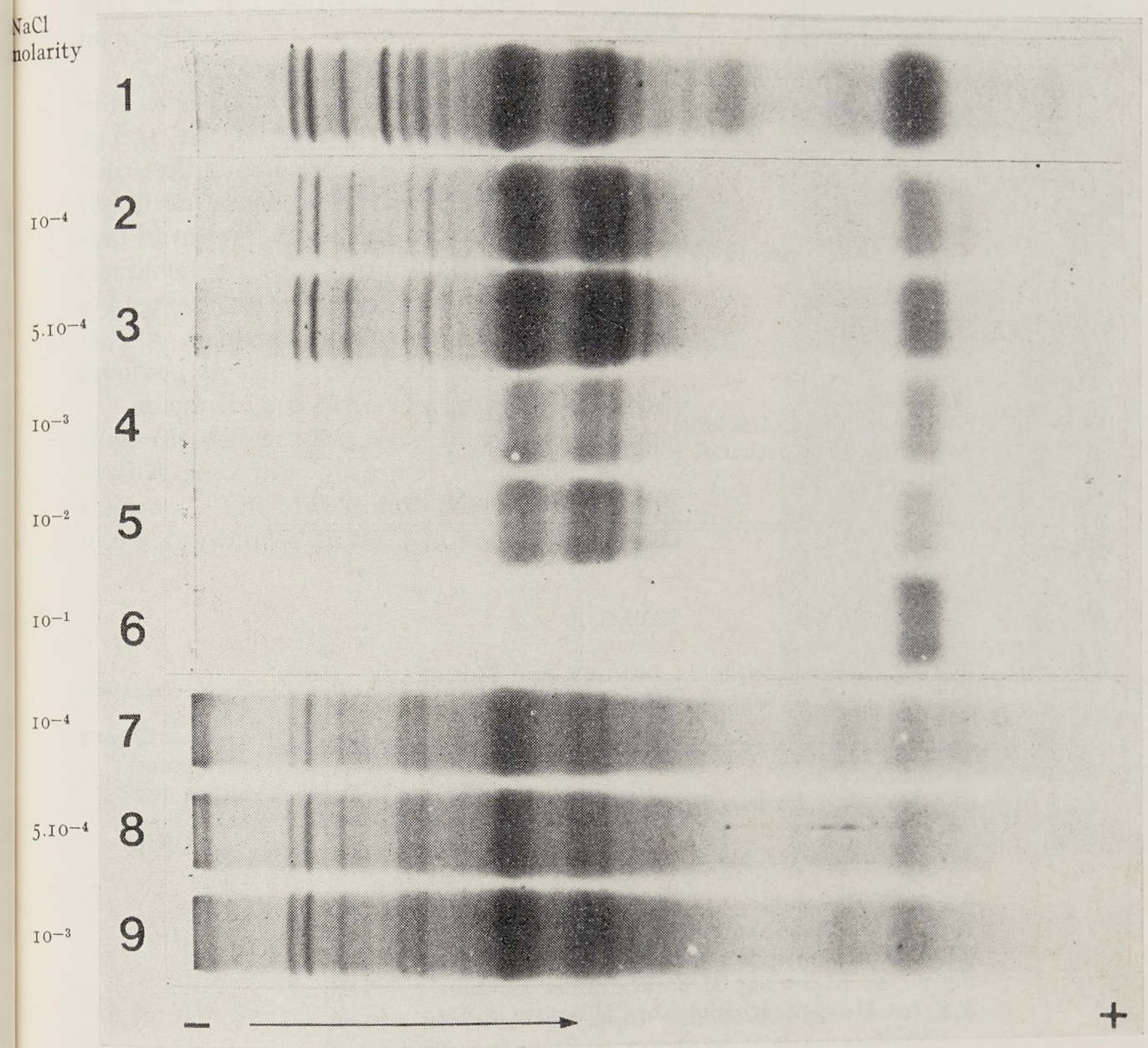


FIG. 7. — *Tris SDS glycine (pH = 8.3) patterns of reduced samples : 1, WSG ; 2 to 6, NaCl soluble fractions ; 7 to 9 NaCl insoluble fractions.*

According to these results it is postulated that WSG protein could possibly consist of three different aggregats of subunits. Such as:

— the one that was present in the 10^{-4} M NaCl insoluble fraction;
— the one that was present in the 10^{-2} or 10^{-3} M NaCl soluble fraction;
— and the one that was present in the 10^{-1} M NaCl soluble fraction.

Further experiments should be carried out in order to verify this hypothesis.

Water insoluble gel protein

By sequential extraction (Table 2) with water, ethanol and 2-chloroethanol only 32.9 p. 100 of the total protein of the WIG were extracted. When the same extraction procedure was applied to WIG before freeze-drying, the total solubility increased to 68.1 p. 100. Thus freeze-drying had reduced, in a large part,

TABLE 2

WIG protein solubility in FEILLET et al. (1977) sequential extraction procedure Results expressed in p. 100 of total protein

Protein fractions	Water insoluble gel	
	Non freeze-dried	Freeze-dried
Water soluble	20.0	8.0
60 p. 100 Ethanol soluble. . .	12.6	7.9
70 p. 100 Chloroethanol soluble	35.5	17.0
Insoluble	31.9	67.1

TABLE 3

WIG protein solubility in LANDRY and MOUREAUX (1970) *sequential extraction procedure*

Protein Fractions	% Total Protein
0.5 M NaCl soluble	8.1
60 p. 100 ethanol soluble.	7.1
0.025 M borate buffer soluble	11.6
0.5 p. 100 SDS-0.025 M borate buffer soluble.	18.8
1 p. 100 Mercaptoethanol-0.025 M borate buffer soluble	51.4
Insoluble	3.0

the solubility. During freeze-drying the gel protein may have been altered in some way to change the solubility properties of its components. Interactions between protein and non-protein material could be involved in these modifications. Solubility of the water soluble gel was not at all, if any, affected by freeze-drying.

We observe large differences (Fig. 8) between the sodium dodecyl sulfate electrophoretic pattern of the water soluble fraction extracted either before or after freeze-drying of the WIG. Water soluble fraction extracted after freeze-

drying had a similar pattern to the WIG. Water soluble fraction extracted before freeze-drying consists mainly of low molecular weight subunits. It is interesting to observe that these subunits were not present in the pattern of the reduced WIG. We can explain that by the presence in the sodium dodecyl sulfate pattern of reduced sample of subunits that were not protein monomers but could be aggregats.

The second sequential extraction procedure used (Table 3) allow the solubilization of 45.6 p. 100 of total protein before extraction with reducing agents. By comparison with the first procedure, the SDS borate buffer step improved the solubility slightly. But a complete solubilization of the protein required extraction with mercaptoethanol. These results suggest that disulfide bonds were involved in gel protein structure.

According to the electrophoretic study (Fig. 9), differences were observed between soluble fractions obtained by sequential extraction. The lowest molecular weight subunits only occured in the NaCl-soluble fraction.

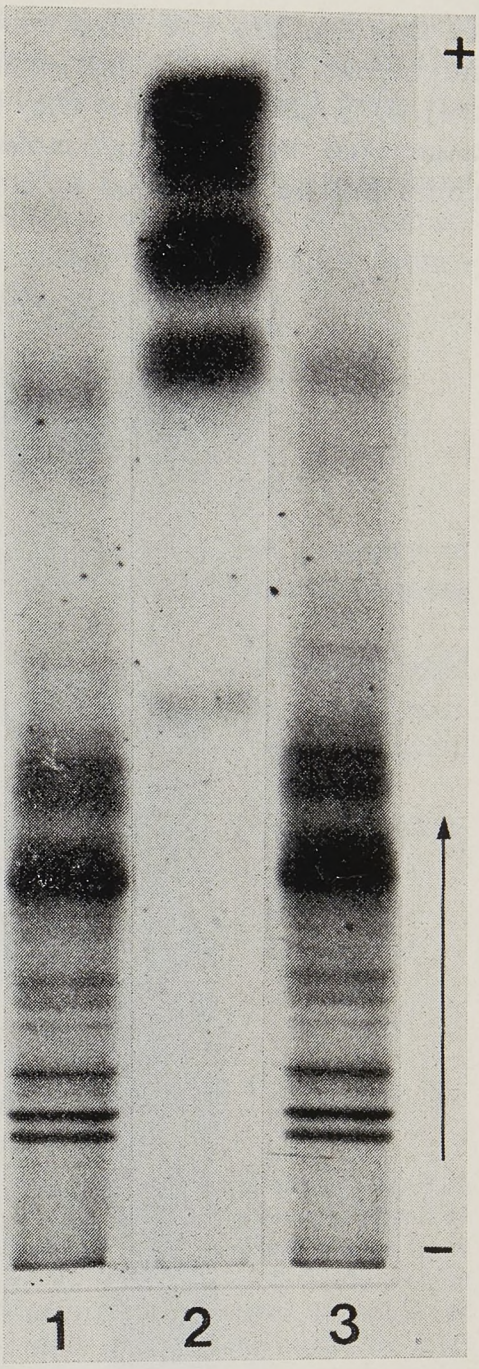


FIG. 8. — *Tris SD Sglycine (pH = 8.3) patterns of reduced samples : 1, WIG ; 2, water soluble fraction extracted before freeze-drying of WIG ; 3, water soluble fraction extracted after freeze drying of WIG.*

TABLE 4

WIG protein solubility in sodium stearate and sodium palmitate
Results expressed in p. 100 of total protein

Soap concentration g/100 gel protein	Sodium Stearate	Sodium Palmitate
2	18.2	14.4
5	49.8	35.3
7.5	73.2	63.4
10	76.8	75.2
12.5	—	84.7
15	73.0	71.3

In this fraction the high molecular weight subunits were missing. The most important fact was that even after extraction by NaCl, ethanol, borate buffer and SDS borate buffer, the medium molecular weight subunits were always associated with the high molecular weight subunits in the insoluble fraction and could not be considered as contaminants.

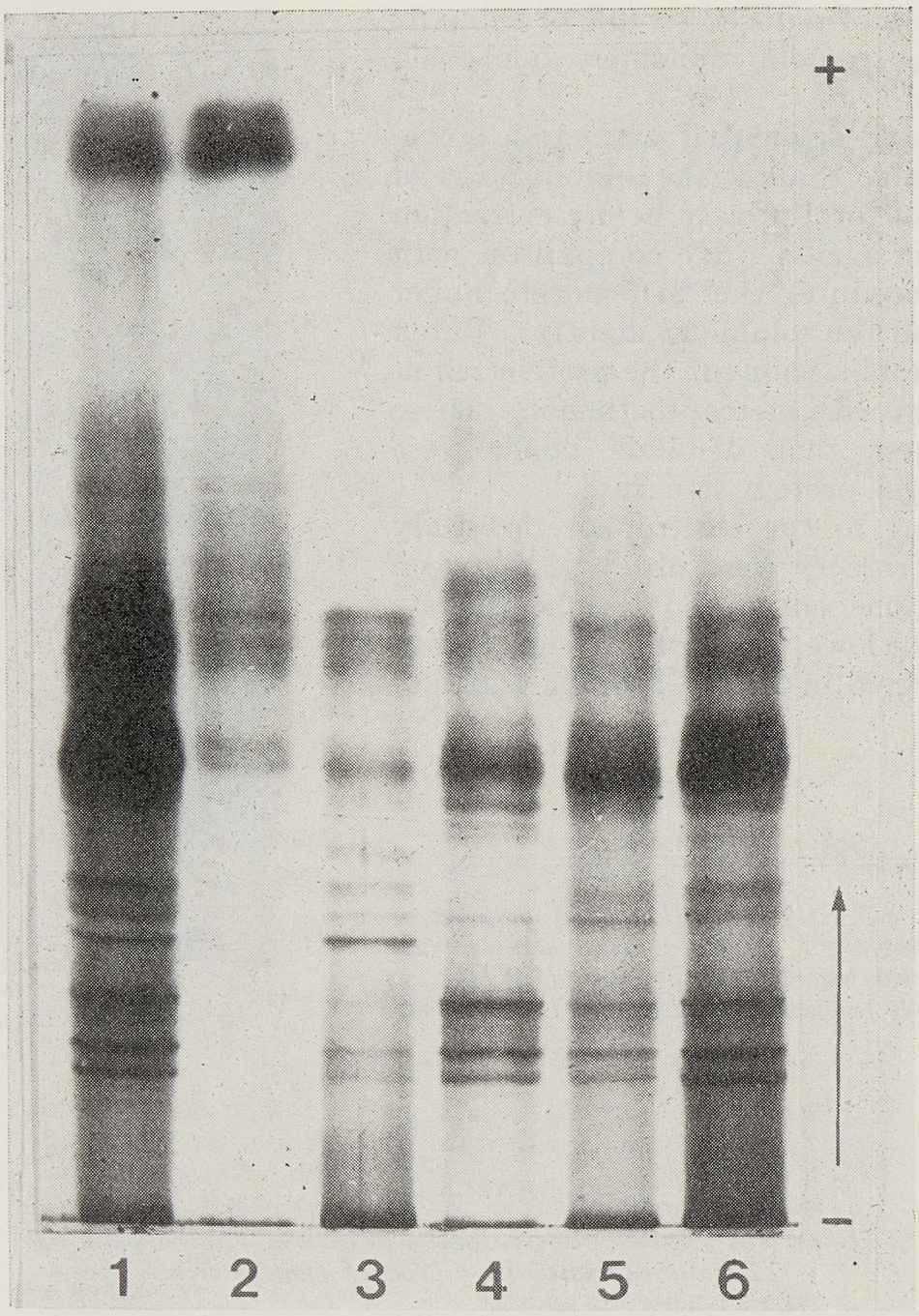


FIG. 9. — *Tris SDS glycine (pH = 8.3) patterns of protein fractions of Table 3 : 1, WIG ; 2, NaCl-soluble fraction ; 3, Ethanol-soluble fraction ; 4, Borate-buffer soluble fraction ; 5, SDS-borate buffer soluble fraction ; 6, Mercaptoethanol-borate buffer soluble fraction.*

These two classical extraction procedures lead us to think that disulfide bonds were involved in the gel protein structure. However according to a recent work by KOBREHEL and BUSHUK (1977) on glutenin solubilization by soaps, further experiments were conducted. As shown in Table 4, after only one extraction 84.7 p. 100 and 76.8 p. 100 of total protein of the WIG were respectively

solubilized by Na-palmitate and Na-stearate. Concentrations study here were not necessarily optimum concentrations. Thus, soaps appeared as very effective to solubilize WIG by comparison with the other solvents used, except reducing agents as mercaptoethanol.

Recent studies (HAMAUZU *et al.*, 1979; WASIK *et al.*, 1979) have underlined that sodium acid salts did not disrupt disulfide bonds but were able to dissociate protein organized through non covalent bonds. According to these results, bonds involved in the WIG were mainly non covalent bonds.

Conclusion

Gel filtration results and salt sensitivity study underlined the capacity of the WSG proteins to aggregate. Their high degree of association makes more difficult purification attemps.

The high molecular weight subunits of WSG and WIG proteins were associated with the medium molecular weight subunits even after fractionation procedures.

According to solubility in soaps bonds involved in the native gel protein were mainly non covalent types.

Freeze-drying can induce strong modifications in the structure and consequently in the properties of gel protein.

Reçu pour publication en octobre 1980.

Résumé

Propriétés du gel protéique

Le gel protéique dont la teneur est reliée à la force boulangère des blés correspond à la fraction protéique présente dans le résidu fortement hydraté apparaissant après extraction de la farine par l'eau et le 2-chloroéthanol. Le gel protéique est séparé, après dialyse contre l'eau désionisée, en gel soluble dans l'eau et gel insoluble dans l'eau. Le gel soluble est fractionné en deux pics par gel filtration sur Bio Gel P 60 ou Sephacryl S 200. Les sous-unités de faibles et moyens poids moléculaires sont présentes dans les deux pics alors que les sous-unités de haut poids moléculaire sont seulement présentes dans le premier pic. La solubilisation dans des solutions de NaCl (0 à 0.1 M) montre que les sous-unités de haut poids moléculaire, du gel soluble, sont les premières insolubilisées quand la force ionique augmente. Alors qu'une grande partie des sous-unités de poids moléculaire moyen sont encore solubles à la même force ionique.

Le gel insoluble dans l'eau est fractionné par des extractions séquentielles. Des études de solubilisation par les savons suggèrent que les liaisons impliquées dans la structure native du gel ne sont pas de type covalent.

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The high-molecular-weight subunits of glutenin : structure, genetics and relationship to bread-making quality

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Summary

Glutenin was prepared by gel filtration chromatography and its subunits were characterised after reduction by SDS-PAGE. A group of high-molecular-weight (HMW) subunits of nominal molecular weight between 95 000 and 140 000 were selected for further study. Using nullisomic, monosomic, telocentric and inter-varietal chromosome substitution lines it was shown that all the HMW subunits were coded by the homoeologous group I chromosomes. These chromosomes contain in total 5 major gene groupings which code for the HMW subunits; two occur on the long arm of chromosome 1D, two are on chromosome 1B and one on chromosome 1A. Each gene group displays allelic variation that is detectable by SDS-PAGE. Some of the different allelic subunits have been assessed for their relationship to bread-making quality. Two strong correlations with quality were detected in the progeny of several crosses: the presence of subunit 1, a polypeptide coded by genes on chromosome 1A, and the presence of subunits 5 and 10, coded for by chromosome 1D. The results are discussed in relation to future strategies for breeding wheats with improved bread-making quality. Numerous species of *Aegilops* and *Triticum* have been screened for HMW polypeptides by SDS-PAGE. One of the polypeptides in *Aegilops umbellulata* has been partially characterised. The genes which control its synthesis on the 1Cu chromosome are being transferred to the chromosomes of Chinese Spring.

Key words : *wheat — glutenins — composition — genetics — breadmaking quality.*

Introduction

It is generally accepted that the properties of the proteins in a flour govern its suitability for processing into bread. This " protein quality " has principally been linked to the glutenin proteins: the bread-making quality of a flour is directly proportional to (1) the amount of glutenin insoluble in 3M urea (POMERANZ, 1965) or dilute acetic acid (ORTH and BUSHUK, 1972) and (2) the mean molecular weight of total glutenin (HUEBNER and WALL, 1976). Glutenin forms about 45 p. 100 of the total endosperm protein and consists of several different subunits (HUEBNER and WALL, 1976) which are connected together to form high-molecular-weight complexes. The subunit composition of glutenin varies according to the

wheat variety selected (BIETZ *et al.*, 1975) so different combinations of subunits could account for the different properties of glutenin, which, in turn, could cause the differences in bread-making quality of flours.

On the basis of evidence in the literature (see discussion of PAYNE *et al.*, 1979) the high-molecular-weight (HMW) subunits of glutenin have been selected

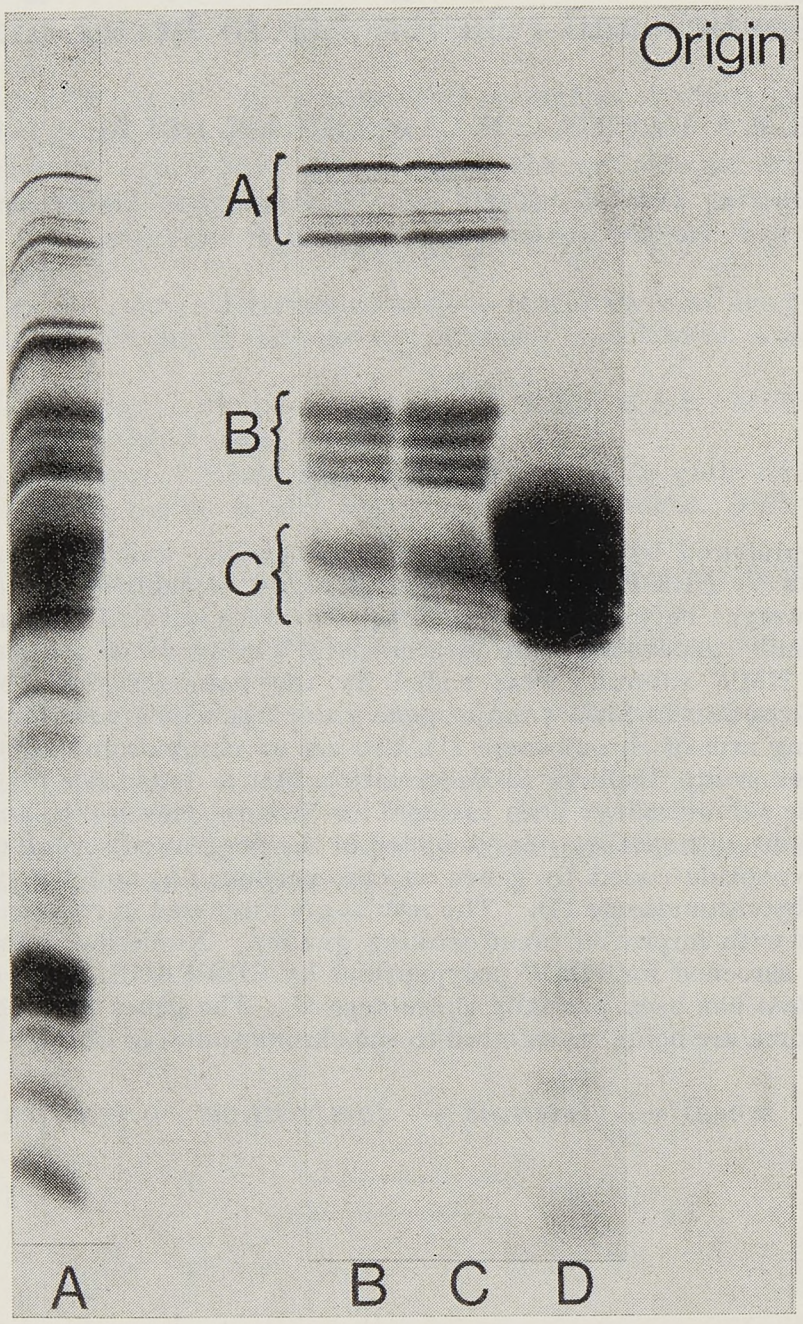

FIG. 1. — *SDS-PAGE of protein fractions from the variety Mardler. Slot A, total proteins of white flour; slots B and C, subunits of glutenin prepared by gel-filtration chromatography with Sepharose 4B-CL; slot D, classical gliadin prepared by gel-filtration chromatography of aqueous alcohol-soluble proteins with Sephadex G-100, overloaded to reveal minor components. The A, B and C subunits of glutenin have been grouped as shown.*

for the initial study. After a brief account of the characterisation of subunit types in glutenin prepared by gel filtration chromatography, the genetics of the HMW subunits are described and this is followed by a progress report of the results obtained so far in determining the relative roles of the different HMW subunits in bread-making quality.

Materials and methods

Plant material

The Koga II aneuploid lines (nullisomic 1D, monotelocentric 1D and ditelocentric 1D) were all developed at the Plant Breeding Institute, Cambridge, U. K., by recurrent backcrossing of Koga II to the relevant monosomic and ditelocentric lines of Chinese Spring and using the cytological selection methods described by Law and Worland (1973). The two telocentric lines were test crossed to verify their aneuploid status.

SDS-polyacrylamide Gel Electrophoresis (SDS-PAGE)

Either 14 or 20 samples were fractionated on each polyacrylamide gel by the method of Laemmli (1970) with modifications as described by Payne *et al.* (1980).

SDS-Sedimentation Test

Wholemeal flour samples were tested for bread-making quality by the SDS-sedimentation test of Axford *et al.* (1978). In this method, the volume of material which sediments after mixing flour with a solution of SDS and lactic acid is measured. Axford *et al.* (1979) have shown that sedimentation volumes of the flours of 56 varieties correlated strongly with the volume of loaves made from the same flours by both a long fermentation baking process and a mechanical development baking process.

Starch-gel electrophoresis

Proteins soluble in 25 p. 100 2-chloroethanol were fractionated in slabs of starch gel (15 samples /slab) using a solution of aluminium lactate as the electrophoresis buffer. The apparatus and the conditions of electrophoresis used were similar to those described by Autran (1979).

Results and discussion

1. — *Subunit composition of glutenin prepared by gel-filtration chromatography*

Glutenin, unlike gliadin and other proteins of the endosperm, has a high molecular weight (Meredith and Wren, 1966). Consequently, it is excluded from all types of Sephadex column and provided a highly dissociating medium is used to prevent protein-protein interaction, it can be recovered in a pure state. In most of our studies (Payne and Corfield, 1979), we have used Sepharose CL rather than Sephadex to obtain a better subfractionation of glutenin and AUC

for both protein extraction and column equilibration. The subunit composition of glutenin prepared in this way is shown in Figure 1B-C. Twelve major components separate and they fall into three groups; the A subunits have molecular weights of between 136 000 and 95 000, the B subunits, 51 000-42 000 and the C subunits 35 500-31 500. The complexity of the subunit composition is much less than that found for glutenin prepared by differential solvent extraction. For instance, BIETZ *et al.* (1975) in addition to the subunits found here also detected components of molecular weight inbetween those of the A and B subunits and components of lower molecular weight than the C subunits. These are all probably components arising from incomplete extractions of the water-soluble, salt-soluble and aqueous-ethanol soluble proteins prior to the extraction of glutenin.

Figure 1D shows a fractionation of gliadin, purified by gel-filtration chromatography to eliminate HMW gliadin and small albumins that are soluble in both water and aqueous ethanol. The gel slot was purposely overloaded to reveal any minor components. There are no polypeptides of similar molecular weight to the A subunits of glutenin and only trace amounts of polypeptides similar in mobility to the B subunits. The bulk of the gliadins migrate either more slowly than the C subunits or at the same rates and correctly-loaded separations (not shown) show a much more complex banding pattern. It is not known if the C subunits of glutenin are identical to some of the gliadin components. As shown by CORFIELD and PAYNE (1979), HMW gliadin consists of subunits which have identical mobilities to the B and C subunits of glutenin so the alternative name low-molecular-weight glutenin suggested by BECKWITH *et al.* (1966) seems most appropriate for them.

The A subunits have different biochemical properties from the B and C subunits (BIETZ and WALL, 1973) and as there are indications in the literature that the A subunits are primarily responsible for producing the type of glutenin that is required for breadmaking (see PAYNE *et al.*, 1979) these polypeptides have been studied first. In subsequent sections, the A subunits are described as high-molecular-weight (HMW) subunits of glutenin.

2. — *Genetics: determination of the chromosomes which code for the HMW subunits of glutenin*

In a previous communication (PAYNE *et al.*, 1980), the electrophoretic mobilities of the HMW subunits of glutenin from 7 varieties were compared by SDS-PAGE. In total, 12 subunits were resolved and the chromosomes which control their synthesis were determined using monosomic lines and inter-varietal substitution lines. All subunits were shown to be controlled by the homoeologous group 1 chromosomes. Each variety contained between 3 and 5 subunits; 2 were under the control of the 1D chromosome, 1 or 2 were controlled by chromosome 1B and 0 to 1 by chromosome 1A.

As a continuation of this work, nullisomic and telocentric lines have been analysed by SDS-PAGE. Figure 2 shows the results obtained with Koga nulli 1D, Koga monotelocentric 1D and Koga ditelocentric 1D. Subunits 5 and 10 of the variety Koga are completely missing in Koga nulli 1D (Fig. 2A), a genetic stock which has completely lost its complement of 1D chromosomes. This shows that the genes which control the synthesis of subunits 5 and 10 are located on chromosome 1D. Grains which lack both of the short arms of each pair of 1D chromosomes and contain only one of the long arms (Koga monotelocentric 1D)

did contain subunits 5 and 10 but in depleted amounts (Fig. 2B). In contrast, subunits 5 and 10 occurred in Koga ditelocentric 1D (short arms missing, but the two 1D long arms present) at the same dosage as in the Koga euploid (Fig. 2C). This shows that the genes controlling the synthesis of subunits 5 and 10 are located on the long arm of chromosome 1D. In another experiment the gliadin proteins extracted from the same genetic stocks were fractionated by starch gel electro-

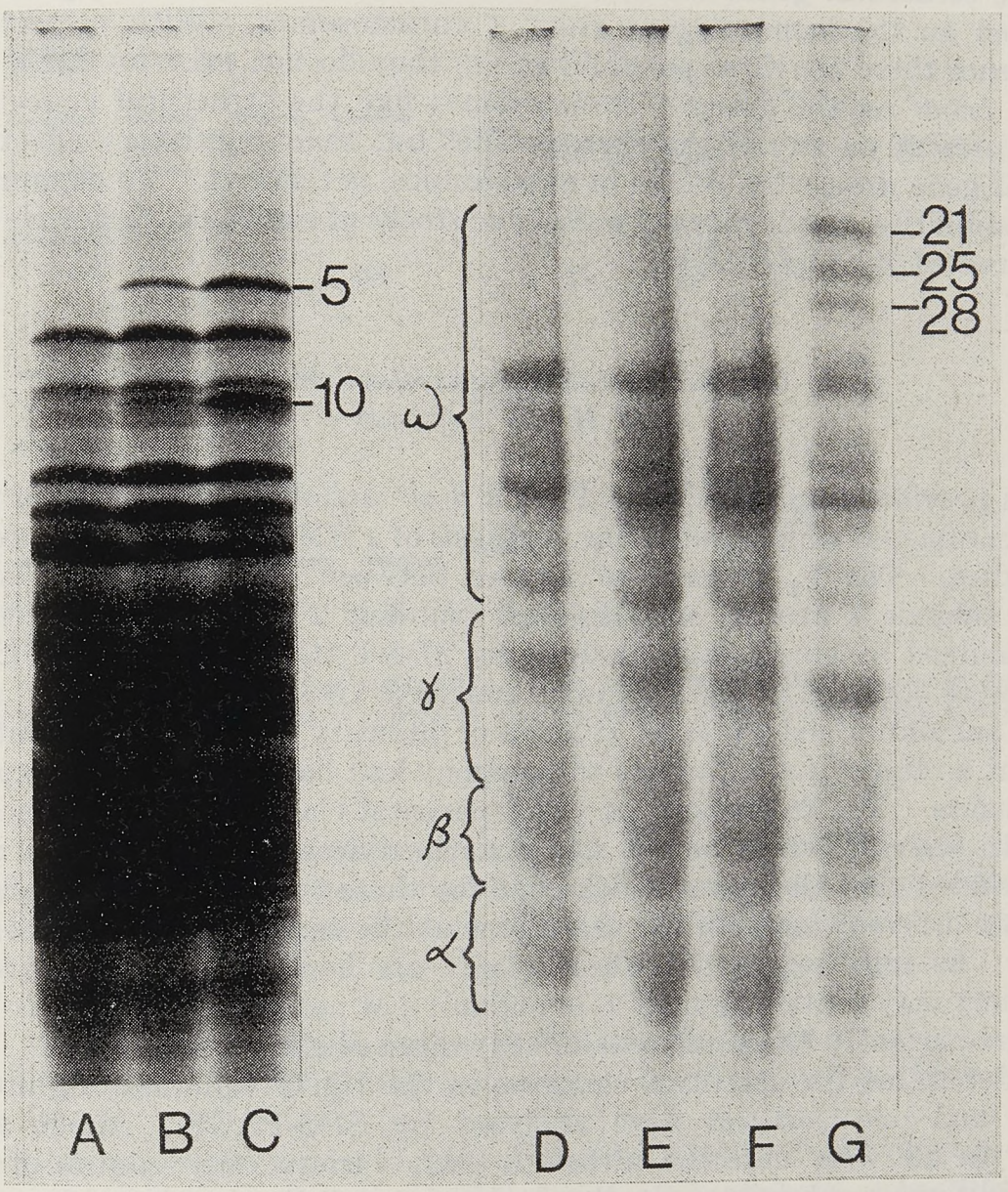

FIG. 2. — *SDS-PAGE of total protein (slots A-C) and starch gel electrophoresis (slots D-G) of gliadins from Koga II (slot G), Koga 1D nullisomic (slots A and D), Koga 1D monotelosomic (slots B and E) and Koga 1D ditelosomic lines (slots C and F). Subunits 5 and 10 are marked by the SDS-PAGE separation. The two HMW subunits of intermediate mobilities to 5 and 10 are present at the same dosage in all three genetic lines and are coded by chromosome 1B.*

phoresis using aluminium lactate as the electrophoresis buffer. Three of the major ω-gliadins found in Koga euploid (Fig. 2G) and numbered 21, 25 and 28 by the scheme of ELLIS and BEMINSTER (1977) were completely lacking in all three genetic lines (Fig. 2 D-F), showing that these polypeptides are controlled by genes located on the short arm of chromosome 1D.

The genetic approach described above cannot distinguish between the identity

of structural genes for the HMW subunits of glutenin and regulatory genes which indirectly affect subunit synthesis. In further experiments, 4 grains from each of 20 of the possible 21 monosomic lines of Hobbit sib, a sister line of Hobbit, were analysed by SDS-PAGE on a single grain basis. Progeny of chromosome 3D monosomic grain of Hobbit sib were unfortunately not available. The intensities of the HMW subunits in polyacrylamide gels after staining were only reduced in some of the progeny of chromosome 1B and chromosome 1D monosomic grain. Thus the structural genes which code for the HMW subunits of glutenin must be located on the homoeologous group 1 chromosomes. With respect to regulatory genes there are three possibilities: (1) they do not exist for these subunits; (2) they occur on the group 1 chromosomes like the structural genes; (3) they may be present on any of the chromosomes but their regulatory effects are not altered if gene dosage (as occurs in monosomics) is reduced. To examine further these possibilities, a wide range of nullisomic stocks of various varieties are currently being screened by SDS-PAGE.

3. — *Genetics: establishment of allelism amongst the HMW subunits*

In a previous communication (PAYNE *et al.*, 1980) the proximity of the genes on chromosome 1D which control the synthesis of 2 HMW subunits, termed 2 and 5, was studied. The F_2 progeny of crosses between Chinese Spring (subunit 2 present, subunit 5 absent) and Holdfast (subunit 2 absent, subunit 5 present) were examined by single-grain analysis by SDS-PAGE. Four types of banding pattern were obtained in the 138 grains analysed: (1) subunit 2 only, (2) subunit 5 only, (3) subunits 2 and 5 present at levels of intensity 2:1, and (4) subunits 2 and 5 present at a ratio of 1:2. None of the progeny had banding patterns expected for recombinants. The frequencies of the 4 parental classes above were scored and the results were shown to be not significantly different from a 1:1:1:1 ratio. It was concluded that the genes which code for these two subunits are allelic, that is they are different forms of the same gene at the same position on the 1D chromosome. As fully segregated wheat plants are homozygous, it follows that a pure variety may contain subunit 2, or subunit 5 or neither but not both. Further crosses are currently being made to identify other allelic variants.

To determine the extent of variation in the HMW subunits of glutenin, one hundred and one varieties were analysed by SDS-PAGE. About 20 major subunits in all were detected although each variety only contained between 3 and 5 different subunits. On the basis of previous genetic analyses (PAYNE *et al.*, 1980) the genes for all the subunits could be confidently assigned to specific chromosomes. Accordingly the subunit variants have been split into three groups (Fig. 3): variants coded for by chromosome 1A, by chromosome 1B and by chromosome 1D. Variation was greatest amongst subunits coded for by chromosome 1B and least for those coded by chromosome 1A. Some of the subunit variants were much more common in the range of varieties analysed than others, as is indicated in Figure 3. Each hexaploid wheat variety consists of one chromosome 1B variant-type, one 1D variant-type and either zero or one of the 1A variants. Only rarely was more than one subunit variant type per chromosome observed and in these cases the grain stocks were shown by single-grain analysis to be either heterozygous or of mixed genotypes.

The above survey of HMW subunits of glutenin in a range of wheat varieties

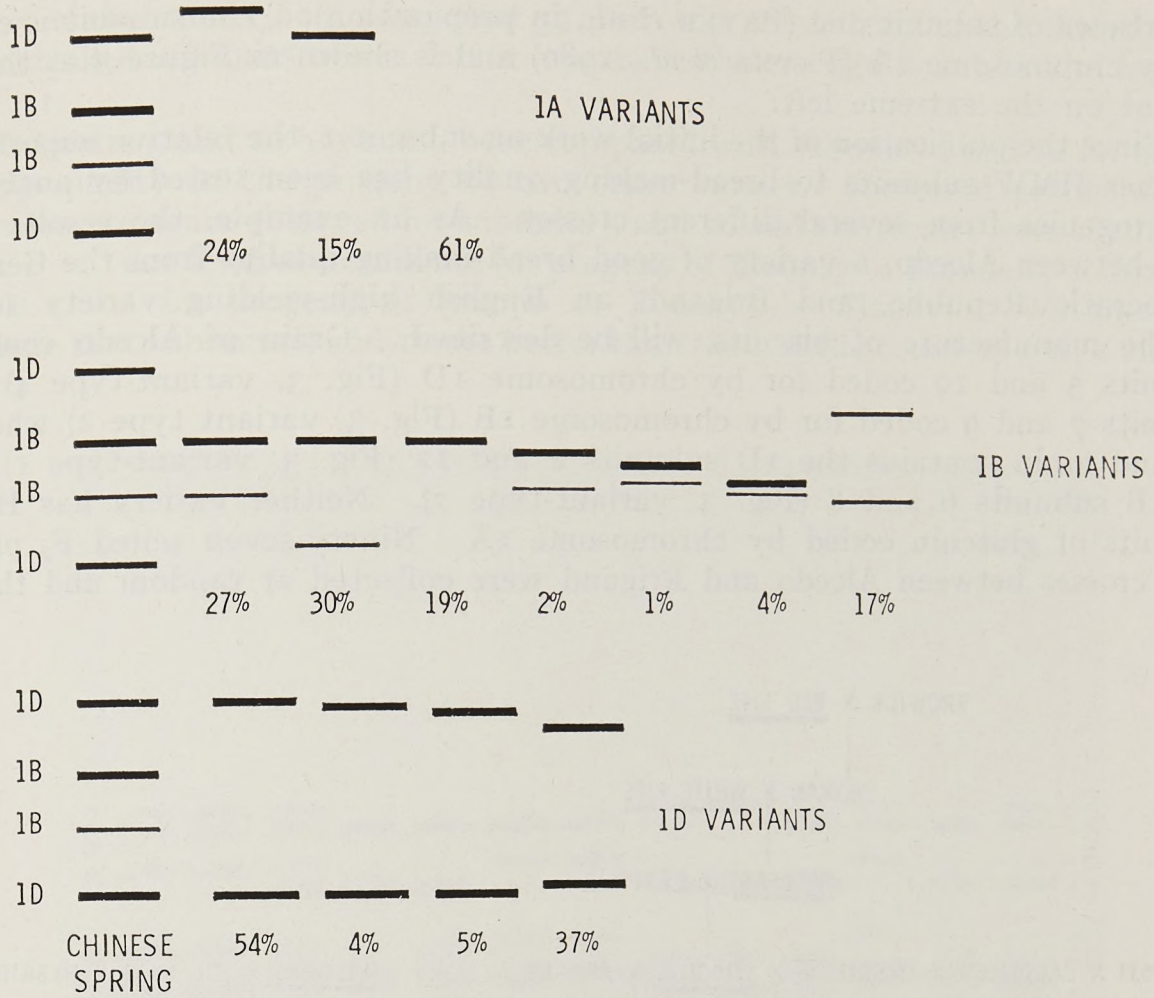

FIG. 3. — *Variation in the banding patterns of HMW subunits of glutenin found in 101 varieties. The frequency of each variant is expressed as a percentage of the total for each group 1 chromosome.*

srrongly suggests that chromosomes 1B and 1D each contain two major loci for these polypeptides whereas chromosome 1A contains only one locus, so that the numerous subunits detected have arisen by allelic variation at these 5 loci.

4. — *Determination of the relative importance of the different HMW subunits to bread-making quality*

It was shown by PAYNE *et al.* (1979) that an HMW subunit of glutenin called subunit 1 was present in all but one of the winter wheats bred at the Plant Breeding Institute, Cambridge, U.K., for breadmaking quality but was absent in all wheats bred for either biscuit-making or animal feeds. In the breeding programme for bread-quality wheats a variety of good quality was invariably crossed with a poor-quality variety which had other important attributes such as high yield and disease resistance. Subunit 1, originating from the good-quality parent was always inherited by those progeny that were selected by the breeder for bread-making quality. This is seen clearly when the pedigree lines of these quality wheats are constructed (Fig. 4). The varieties underlined are those which both contain subunit 1 and possess good bread-making quality. PAYNE *et al.* (1979) also showed that the same subunit was inherited preferentially by those progeny of a cross between Maris Ranger and Maris Widgeon which also inherited bread-making quality. Subsequent work involving 5 other crosses has confirmed the

importance of subunit one (PAYNE *et al.*, in preparation). The subunit is coded for by chromosome 1A (PAYNE *et al.*, 1980) and is shown in Figure 3 as the 1A variant on the extreme left.

Since the publication of the initial work on subunit 1, the relative importance of other HMW subunits to bread-making quality has been tested by analysing the progenies from several different crosses. As an example, the results of a cross between Alcedo, a variety of good bread-making quality from the German Democratic Republic, and Brigand, an English high-yielding variety suited for the manufacture of biscuits, will be described. Grain of Alcedo contains subunits 5 and 10 coded for by chromosome 1D (Fig. 3, variant-type 4) and subunits 7 and 9 coded for by chromosome 1B (Fig. 3, variant type 2) whereas Brigand grain contains the 1D subunits 2 and 12 (Fig. 3, variant-type 1) and the 1B subunits 6 and 8 (Fig. 3, variant-type 7). Neither variety has HMW subunits of glutenin coded by chromosome 1A. Ninety-seven selfed F_2 plants from crosses between Alcedo and Brigand were collected at random and the F_3

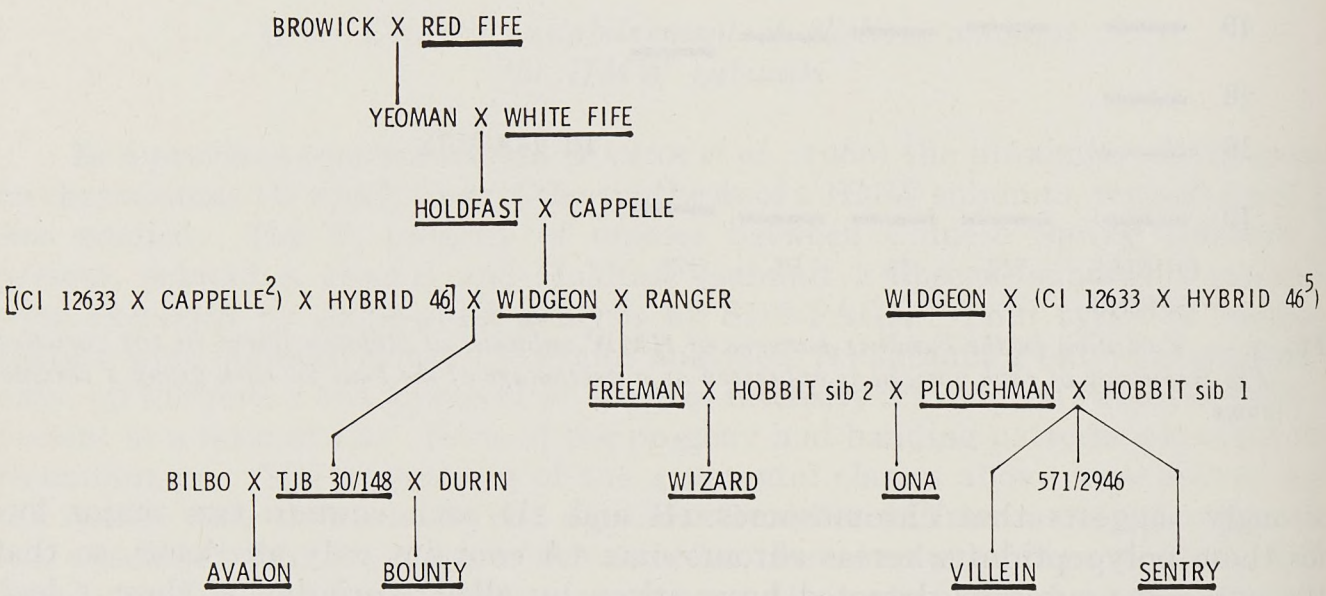

FIG. 4. — *Pedigree lines of winter wheats of bread-making quality, bred at the Plant Breeding Institute, Cambridge, U.K. Varieties underlined are those which possess subunit 1 glutenin.*

grain from each plant was harvested separately and then milled. Each milled sample was tested for bread-making quality by the SDS-sedimentation test and for subunit composition by SDS-PAGE. The subunit composition of a range of progeny screened by SDS-PAGE is shown in Figure 5. The mean sedimentation volume of progeny which possess subunits 5 and 10 but lack subunits 2 and 12 is 70.3 ml whereas the mean sedimentation volume of progeny with subunits 5 and 10 absent and subunit 2 present is less, 57.2 ml. Application of the Student's t test shows that the difference between these two means is significant at the $P < .001$ level of confidence. Those progeny which had a mixture of all 4 subunits had an intermediate, mean sedimentation volume (66.6 ml). A tight genetic linkage was observed between subunits 5 and 10 and it was not broken in any of the 97 progeny analysed. Consequently it is not known whether subunit 5 or subunit 10 or indeed both are beneficial for improved bread-making quality. Subunits 7 and 9 were also shown to be associated with larger sedimentation volumes when compared with subunits 6 and 8. The difference between

the two mean values (69.3 ml against 59.4 ml) was not so large as the chromosome 1D-controlled subunits above but it was nevertheless significant at the level of $P < .01$.

Subunits 5 and 10 have also been compared with subunits 2 and 12 for bread-making quality in the progeny of two other crosses. Increased sedimentation volumes were again observed for subunits 5 and 10 and the results were significant at the $P < .001$ level of confidence for progeny of (Bounty × Flanders) × Armada and $P < .02$ for progeny of (Sappo × Bounty) × Flanders. We therefore conclude that subunits 5 and 10 are better than their allelic counterparts, 2 and 12,

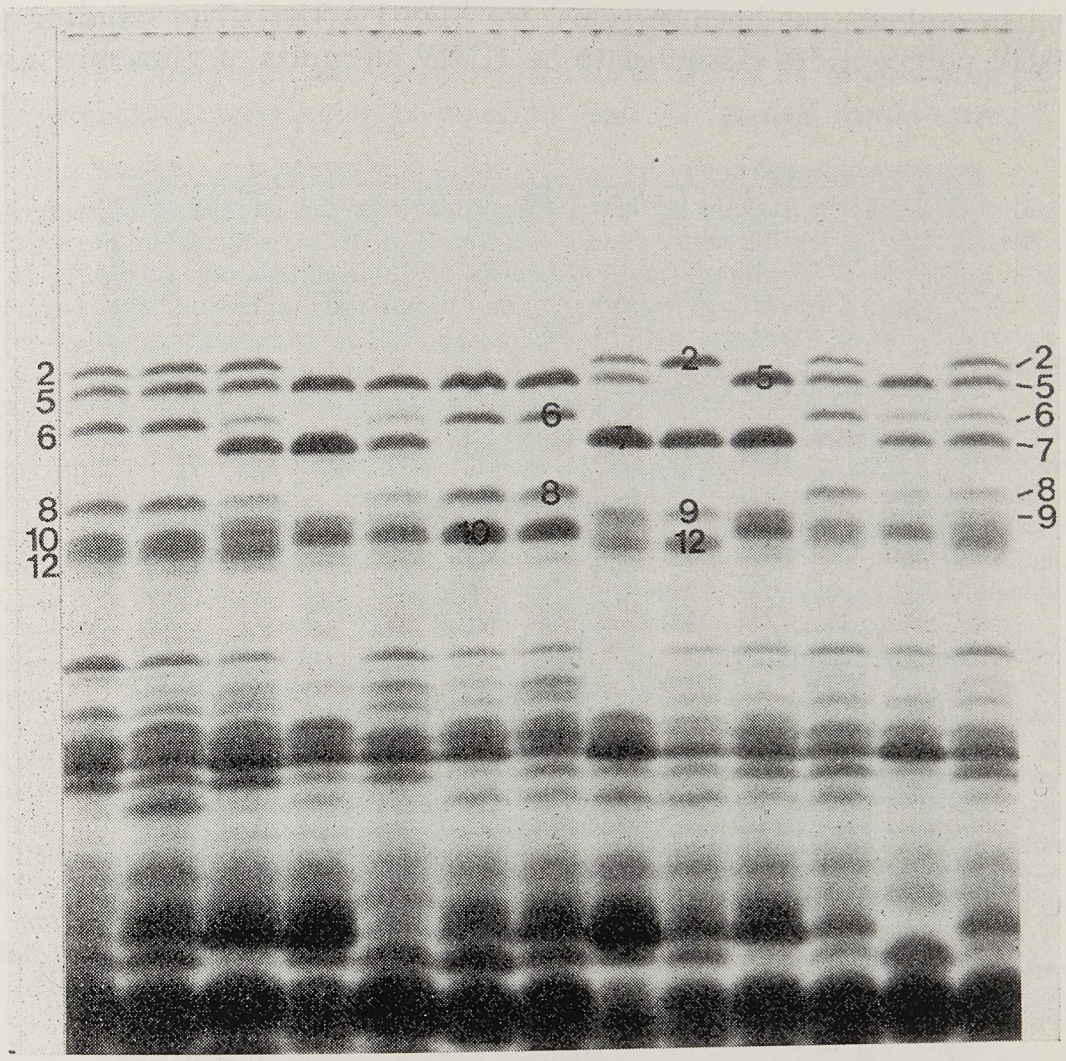

FIG. 5. — *SDS-PAGE of the flours of some F_3 progeny of Alcedo × Brigand.*

at conferring to glutenin the properties required to make it suitable for break-making. Unfortunately the 1B-codes subunits, 7 and 9, have not been compared with subunits 6 and 8 in other crosses so no firm conclusions can be made from the results obtained for Alcedo × Brigand.

The work described in this section suggests that some HMW subunits of glutenin are more effective than others at conferring good bread-making quality to a flour. Further work is required to test many more allelic variants by the same approach and this is in progress. The object with respect to the breeding programme is to determine which allelic variant for each of the 5 loci on the group 1 chromosomes correlates most strongly with bread-making quality. These

subunits will then be brought together by conventional breeding methods using the SDS-sedimentation test as a primary screen and SDS-PAGE as a secondary screen to test which of the better-quality progeny possess the desired subunits. Provided the effects of the good-quality subunits from each of the alleles are additive then new varieties of improved quality should result from this approach.

5. — *HMW polypeptides of alien species and their relevance to the breeding programme for bread-making quality*

The grain of numerous species of *Aegilops* and of *Triticum* diploids, tetraploids and hexaploids has been screened by SDS-PAGE. The banding patterns of the HMW polypeptides (assumed to be HMW subunits of glutenin) are much

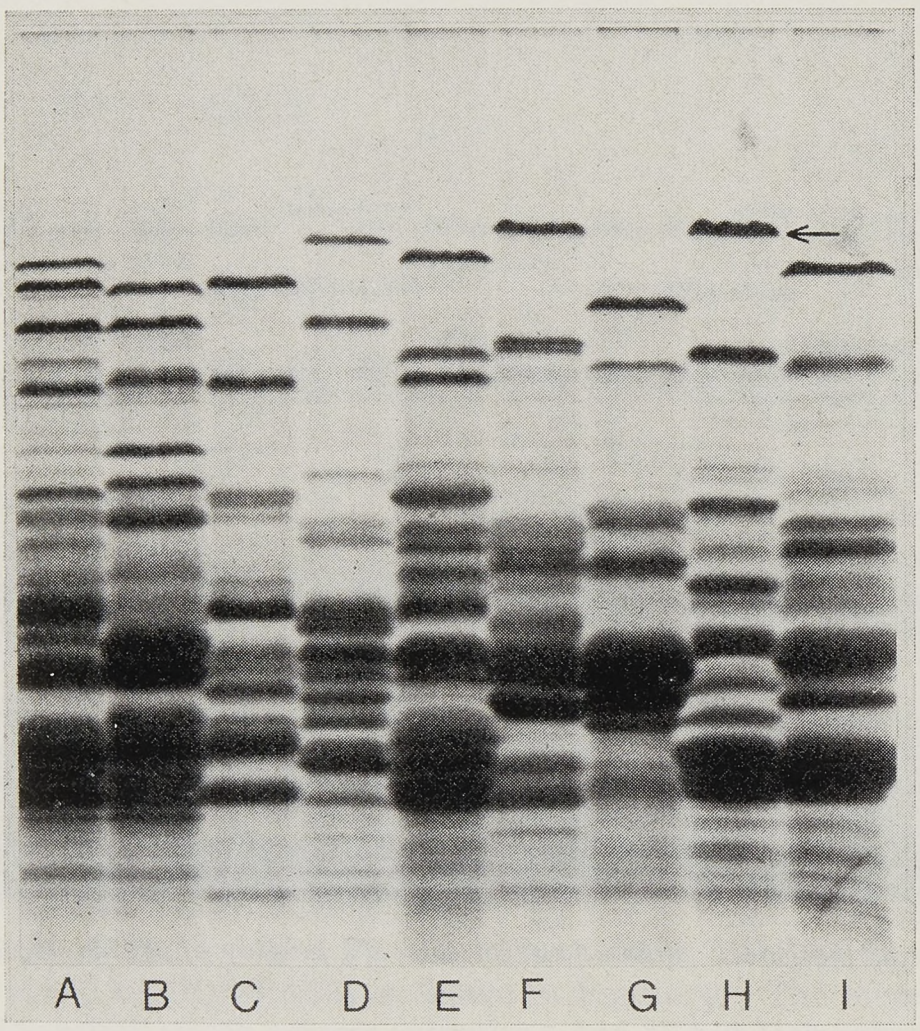

FIG. 6. — *SDS-PAGE of two bread wheats, (A) Bersee and (B) Koga II, and several species of Aegilops; (C) Ae. squarrosa, (D) Ae. longissima, (E) Ae. cylindrica, (F) Ae. bicornis, (G) Ae. speltoides, (H) Ae. umbellulata, (I) Ae. caudata. The Ae. umbellulata subunit referred to in the text is arrowed.*

more varied than those found in bread-wheats (Fig. 6). Since, in antiquity, bread-wheat was selected for characters other than bread-making quality, some of the HMW polypeptides present in alien species may be superior for bread-making purposes to those present in bread wheat. A polypeptide present in *Aegilops umbellulata* has been selected for further study. It has a higher mole-

cular weight than any of the HMW subunits of glutenin in bread wheat, and preliminary results indicate that it falls exclusively into the category of acid-insoluble glutenin. It is also insoluble in AUC at room temperature. The genes which control its synthesis are located on the 1Cu chromosome which is homoeologous to the group 1 chromosomes of bread wheat. Currently these genes are being transferred to the chromosomes of Chinese Spring using a modified version of the procedure developed by RILEY *et al.* (1968).

Résumé

Les sous-unités de poids moléculaires élevés de la gluténine; structure, génétique et relation avec la qualité boulangère.

On prépare la gluténine par chromatographie sur gel Sepharose 4B-CL équilibré en AUC à partir d'extraits protéiques d'endosperme de blé. On caractérise la composition des sous-unités de la gluténine par électrophorèse en gel polyacrylamide-SDS. Environ 12 sous-unités sont nettement résolues et se partagent en 3 groupes; celles du groupe A ont des poids moléculaires compris entre 140 000 et 95 000 approximativement, celles du groupe B entre 51 000 et 42 000 et celles du groupe C entre 35 000 et 31 000. Dans cette communication nous exposerons seulement le travail que nous avons fait sur les sous-unités du groupe A.

Les diagrammes d'électrophorèse en gel de polyacrylamide SDS des sous-unités A ont été établis pour plus de 100 variétés de blé hexaploïdes, et des différences importantes ont été observées bien que chacune des variétés ne contiennent qu'entre 3 et 5 de ces sous-unités.

La localisation de gènes qui contrôlent la synthèse des 12 sous-unités détectées dans 7 variétés, a été déterminée en utilisant des lignées de substitution monosomiques, nullisomiques, telocentriques et inter-variétales. Toutes les sous-unités sont sous le contrôle des chromosomes homologues du groupe I.

L'analyse par électrophorèse de polyacrylamide-SDS de la descendance de différents croisements a montré que le chromosome 1D contient probablement 2 loci principaux pour les sous-unités A et qu'ils sont adjacents. Le chromosome 1B contient aussi deux loci adjacents principaux bien que dans quelques variétés le gène de seulement l'un des loci soit exprimé. Par contre, le chromosome A1 ne contient qu'un seul locus, mais il n'est exprimé que dans moins de la moitié des variétés examinées. Nous envisageons un projet à long terme pour déterminer lequel des variants allèles de chaque locus confère une meilleure qualité boulangère à une farine donnée. Le procédé consiste à croiser deux variétés qui ont des sous-unités A différentes et à analyser la descendance F3 ou F4 pour la qualité boulangère par le test de sédimentation, et pour la composition en sous-unités par électrophorèse en gel de polyacrylamide SDS.

Nous décrivons comme exemple de ce genre de travail des résultats qui suggèrent que deux sous-unités, l'une contrôlée par le chromosome 1A et l'autre par le chromosome 1D présentent une forte corrélation avec la qualité boulangère par rapport à leurs allèles.

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Studies of wheat flour glutenins by modified Landry-Moureaux method and gliadins by isoelectric focusing

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Summary

Wheat flour glutenins (Glu) of 12 samples were extracted by Landry-Moureaux method. Furthermore Glu I, II, III and gliadin (Gli) were separated into water-soluble (S) and -insoluble (Ins) subfractions in the process of dialysis. Percentage of the total N was: Glu I 3.8-6.0, including S 0.4, Ins 3.4-5.6; Glu II 1.5-2.4, including S 1.0-1.6, Ins 0.4-0.8; Glu III 19.6-26.9, including S 17.0-22.7, Ins 2.2-3.6; Gli 43.7-51.7, including S 14.4-23.3, Ins 28.4-30.7. The amino acid analysis showed marked differences which indicated that the glutenin subfractions were of different groups of proteins. Glu I was gliadin-like, but its soluble subfraction was albumin-like (6 p. 100 lysine). Glu II and Glu III had 4.8 and 4.3 p. 100 lysine, 30.7 and 24.8 p. 100 glutamic acid, 9.9 and 10.0 p. 100 proline respectively. Ins Glu II had traces of tyrosine. The amino acid differences between S and Ins gliadins were not significant but isoelectric focusing behaviour was different. The S-Gli components run towards the basic isoelectric points of pH, while the Ins-Gli towards the acidic ones. The varieties differed in number and position of bands. The total spectrum for 4 wheat varieties: S-Gli 55, Ins-Gli 55, S + Ins 65. The analysis of 8 samples of wheat flour showed a positive correlation between Glu III and flour strength and a negative one between S-Glu I, S-Glu II, their total content and flour strength. Positive correlation was higher when the flour strength was compared with such ratios as Ins-Glu I/S-Glu I, Ins-Glu I/S-Glu II.

Rey words : *wheat — gliadin — glutenins — composition — bread-making quality.*

The baking qualities are determined by the composition and physico-chemical properties of gluten. Therefore, two classes of proteins, glutenins and gliadins attract the attention of scientists as basic components of gluten. The application of chromatographic and electrophoretic methods helps to establish the heterogeneity of gliadin and glutenin. For example, WRIGLEY and SHEPHERD 1973 (16) using two-dimensional system (isoelectric focusing and electrophoresis) have separated the gliadins into more than 46 proteins. By sodium-dodecylsulphate gel electrophoresis 15 components were identified in reduced glutenin (1). The reduced glutenin is separated by gel filtration into three distinctly different groups

of polypeptides A, B, C which in their amino acid composition and molecular weight are similar to albumin, high-molecular weight glutenins and gliadins, respectively. The similar protein groups are extracted from reduced maize glutelin by Landry-Moureaux methods (7, 8, 11). However, this method didn't find any apparent application for wheat protein studies.

Paulis and Wall, 1977 (11) found that being isolated from reduced glutelin zein-like proteins had separated into water-soluble and insoluble subfractions. Independently we found that glutelin-like protein and true glutelin of maize divided into water-soluble and insoluble subfractions which differed much in amino acid composition (13). The different water behaviour of reduced glutelins suggested us an idea of advisability of wheat protein studies by Landry-Moureaux method with subsequent dividing into soluble and insoluble subfractions for estimation of their quantity, amino acid composition and their relation to the baking qualities. Moreover, it was of an interest to ascertain the differences between water-soluble and ethanol-soluble gliadins in their amino acid and component composition by isoelectric focusing method.

Materials and methods

Two groups of wheat flour were the objects of our investigation. The first group consisted of varieties Bezostaya 1, Krasnodarskaya 46, Saratovskaya 29, Voskhod. Proteins of this group were submitted to more detailed studies. The second group consisted of varieties Avrora, Rostovtchanka, Deviz, Skorospelka, Rannyaya 12, Kavkaz, Mironovskaya Youbileinaya, Lutescens 520. To calculate correlation coefficients for milling and baking qualities of flour the second group was analysed for fraction composition only.

The flour samples (70 p. 100) were prepared on a " Quadrumat Junior " mill. The quality of gluten was estimated by IDK-1 instrument, flour strength and elastic properties were estimated by " Chopin " alveographe.

Proteins were isolated from three 10 g flour samples per each variety. Aliquots of extracts were assayed for introgen by Kjeldahl method. The scheme is given in Table 1. Protein fractions for amino acid analysis were isolated from 100 g of flour and then lyophilized. About 10 mg of protein were hydrolyzed with 10 ml 6 N HCl for 24 hs at 110 °C. Amino acids were determined by KLA-5-Hitachi analyser. Methionine and cystine were determined by performic acid oxidation. Gliadins were isoelectrically focused on thin-layer polyacrylamide gel by using LKB 2117 Multiphor.

Isoelectric Focusing of Gliadins

Preparation of gel: 21.6 g of urea, 1.8 g of acrylamide, 0.09 g of methylen bis-acrylamide, water to total volume of 56 ml, 3 ml of ampholine. Catalyst is ammonium persulphate-riboflavine. Photopolymerization lasted 2 hrs.

Putting of Protein

The lyophilized protein preparate was dissolved in 8 M urea (protein concentration 5 mg/ml) and 100-150 μl were put on the small square of filter paper which was placed on the surface of the gel at the anode. The filter strips at cathode and anode were soaked with 1 M NaOH and 1 M H_3PO_4 respectively.

TABLE I

Scheme of Extraction

Protein	Solvent	Ratio sample/solvent (W/V)	Time of one extr.	Temp. °C	Method of extraction
Albumin.	NaCl 1 M	*a*) 1st extr. 1:5	16 hs	4	Stand, centrifugation
Globulin.	pH 7 buffer 0.1 M phosphate	*b*) 2nd to 6th 1:10	60 min	4	at 16 000 g
Non-prot. N	pH 7 water	*c*) 7th 1:10	10 min		20 min
Gliadin	Ethanol 70 p. 100	The same " *a* "	180 min	room	The same
		The same " *b* "	60 min	—	
H_2O-soluble		*d*) dialysis against dist. H_2O	48 hrs	4	The same
Insoluble					
Glutenin I.	Ethanol 70 p. 100 + 2-ME 0.6 p. 100	The same " a "	180 min	room	The same
		The same " b "	60 min	—	
H_2O-soluble		*e*) dialysis against water-supply	8 hrs	—	The same
Insoluble		The same " d "	48 hrs	4	
Glutenin II	Borate buffer pH 10 + 2-ME 0.6 p. 100	The same " *a* "	180 min	room	The same
		The same " *b* "	60 min	—	
H_2O-soluble		The same " *e* "	8 hrs	—	The same
Insoluble		The same " *d* "	48 hrs	4	
Glutenin III	Borate buffer pH 10 + 2-ME 0.6 p. 100 + SDS 0.5 p. 100	The same " *a* "	180 min	room	The same
		The same " *b* "	60 min	—	
H_2O-soluble		The same " *e* "	8 hrs	—	The same
Insoluble		The same " *d* "	48 hrs	4	
Scleroprotein. . . .	H_2O-distill.	1st 1:10	30 min	room	The same
	Ethanol 96°	2nd 1:5	30 min	vacuum drying	

Isoelectric Focusing of Water-Soluble Gliadins

The ampholine pH range was 4-9. The quantity of protein was 600 μg. The initial voltage was 520 V, 15.7 mA, the final — 1 200 V, 6.4 mA, the duration — 6 hrs.

Isoelectric Focusing of Ethanol-Soluble Gliadins

The ampholine pH range was 4-9. The quantity of protein was 600-640 μg. The initial voltage was 710 V, 11 mA, the final — 1 300 V, 4.9 mA, the duration — 10 hrs.

Isoelectric Focusing of Total (Sol + Insol) Gliadins

The ampholine pH range was 6-9. The quantity of protein was 600 μg. The initial voltage was 650 V, 13.1 mA, the final — 1 400 V, 5.5 mA, the duration — 7 hrs.

The gels were fixed in 10 p. 100 TCA.

Results and discussion

The different gliadin content in wheat flours depends on the level of water-soluble subfraction. The good baking quality Bezostaya 1 had the lower level of water-soluble gliadin-14.4 p. 100, the poor one Voskhod had the higher — 23.3 p. 100 (Table 2). In addition Voskhod variety had the lowest level of total glutenin and of glutenin I and III subfractions (Table 3).

TABLE 2

The Content of Main Protein Fractions in Wheat Flour (p. 100 of total N)

Item	Bezostaya 1	Krasnodarskaya 46	Saratovskaya 29	Voskhod
Gluten quality (Class)	I	I	II	III
Flour strength (W. alveographe)	366	409	374	55
Resistance (P. alveographe) .	132	118	102	26
Alb + Glob + Non-prot. N .	14.7 ± 0.22	13.7 ± 0.12	13.6 ± 0.24	15.0 ± 0.24
Gliadin	43.7 ± 0.27	46.7 ± 0.13	47.3 ± 0.84	51.7 ± 1.03
H_2O-soluble	14.4 ± 0.78	16.0 ± 0.47	18.7 ± 0.37	23.3 ± 0.64
Ethanol-soluble	29.3 ± 0.86	30.7 ± 0.05	28.6 ± 1.42	28.4 ± 0.87
Glutenin(*)	32.5	32.2	34.1	25.2
Scleroprotein	2.1	2.0	1.3	2.1
Total	92.7	94.6	93.1	91.9

*The glutenin was summarized from glutenin subfractions.

TABLE 3

The Content of Glutenin Subfractions

Fractions	Bezostaya 1	Krasnodarskaya 46	Saratovskaya 29	Voskhod
	p. 100 of total N of wheat flour(*)			
Glutenin I	5.7 ± 0.16	6.0 ± 0.05	5.7 ± 0.13	3.8 ± 0.37
H_2O-soluble	0.4 ± 0.19	0.4 ± 0.07	0.4 ± 0.03	0.4 ± 0.04
Insoluble	5.3 ± 0.19	5.6 ± 0.07	5.3 ± 0.03	3.4 ± 0.04
Glutenin II	1.9 ± 0.08	2.4 ± 0.17	1.5 ± 0.10	1.8 ± 0.21
H_2O-soluble	1.4	1.6	1.1	1.0
Insoluble	0.5	0.8	0.4	0.8
Glutenin III	24.9 ± 0.26	23.8 ± 0.23	26.9 ± 0.23	19.6 ± 0.17
H_2O-soluble	22.7 ± 0.31	20.3 ± 0.64	23.3 ± 0.65	17.0 ± 0.24
Insoluble	2.2 ± 0.31	3.5 ± 0.64	3.6 ± 0.65	2.6 ± 0.24
	p. 100 of total glutenin N			
Glutenin I	17.5	18.6	16.7	15.1
H_2O-soluble	1.2	1.2	1.2	1.6
Insoluble	16.3	17.4	15.5	13.5
Glutenin II	5.8	7.5	4.4	7.1
H_2O-soluble	4.3	5.0	3.2	4.0
Insoluble	1.5	2.5	1.2	3.1
Glutenin III	76.7	73.9	78.9	77.8
H_2O-soluble	69.9	63.0	68.3	67.5
Insoluble	6.8	10.9	10.6	10.3

*Each value represents the mean ± SE for three samples per two analyses with the exception subfractions of glutenin II each of them was determined in one combined sample in two parallel analyses.

At the same time the level of these subfractions of Voskhod variety in percentage of total glutenin did not differ significantly.

The main portion in total glutenin is composed of glutenin III (73.9-78.9 p. 100), including its water-soluble subfraction (63.0-69.9 p. 100). We assume that solubility of this subfraction is caused by association with SDS in spite of dialysis against water. Glutenin II constituted the lowest portion (4.4-7.5 p. 100), glutenin I constituted the intermediate one (15.1-18.6 p. 100).

The amino acid pattern of glutenin I is quite similar to that of gliadin (Table 4). It contains very few of lysine, histidine, arginine but it is rich in glutamic acid and proline. There are some differences between them. Glutenin I contains more

TABLE 4

Amino Acid Composition of Gliadin and Landry-Moureaux Glutenin (g/100 g total amino acids)

Amino-acids	Gliadin	Glutenin I	Glutenin II	Glutenin III
Lys	0.85 ± 0.06	0.97 ± 0.09	4.8 ± 0.29	4.3 ± 0.09
His	1.65 ± 0.03	1.6 ± 0.07	1.8 ± 0.06	2.0 ± 0.05
Arg	1.7 ± 0.08	1.9 ± 0.21	3.8 ± 0.16	4.1 ± 0.15
Thr	2.0 ± 0.14	2.4 ± 0.07	3.3 ± 0.10	3.5 ± 0.13
Asp	2.2 ± 0.07	2.3 ± 0.11	5.8 ± 0.52	5.9 ± 0.15
Ser	4.2 ± 0.06	4.5 ±	4.6 ± 0.17	5.2 ± 0.41
Glu	39.0 ± 0.83	38.6 ± 0.28	30.7 ± 1.56	24.8 ± 0.87
Pro	15.7 ± 0.60	13.8 ± 0.62	9.9 ± 0.31	10.0 ± 0.42
Gly	2.7 ± 0.11	2.6 ± 0.11	7.9 ± 0.26	5.5 ± 0.33
Ala	2.4 ± 0.05	2.7 ± 0.07	4.3 ± 0.17	4.0± 0.12
Cys-Cys	3.1 ± 0.05	3.6 ± 0.24	2.1 ± 0.12	3.2 ± 0.26
Val	3.7 ± 0.07	4.2 ± 0.07	4.1 ± 0.33	4.6 ± 0.12
Met	1.7 ± 0.20	2.4 ± 0.17	1.8 ± 0.09	2.6 ± 0.10
Ile	4.0 ± 0.13	4.2 ± 0.05	2.6 ± 0.22	3.5 ± 0.07
Leu	6.9 ± 0.12	7.1 ± 0.12	5.6 ± 0.24	8.1 ± 0.23
Tyr	2.8 ± 0.18	1.9 ± 0.09	4.2 ± 0.22	3.8 ± 0.20
Phe	5.5 ± 0.13	5.2 ± 0.15	3.0 ± 0.22	4.2 ± 0.15

Each value represents the mean ± SE for 4 varieties.

cystine (3.6 against 3.1 p. 100), methionine (2.4 against 1.7 p. 100) but less tyrosine (1.9 against 2.8) and proline (13.8 against 15.7). It is possible that glutenin I has more cysteic residues on outer-side of the protein molecule than gliadin, therefore, it is associated in glutenin.

Glutenin II and III have more lysine, arginine, threonine, tyrosine but lower quantities of glutamic acid, proline, isoleucine, phenylalanine when compared with gliadin and glutenin I. These proteins resemble more or less albumin and globulin. Glutenin II differs from glutenin III in the more higher level of glycine (7.9 against 5.5 p. 100), glutamic acid (30.7 against 24.8 p. 100). Glutenin II

TABLE 5

Lysine, Proline, Cystine Content in Landry-Moureaux Proteins of Different Wheat Varieties

Amino acids	Bezostaya 1	Krasnodarskaya 46	Saratovskaya 29	Voskhod
Gliadin (total)				
Lys	0.80	0.75	0.85	1.0
Pro	17.0	16.0	14.8	14.9
Cys-Cys	3.1	3.0	3.1	3.2
Glutenin I				
Lys	0.9	0.8	1.1	1.1
Pro	14.7	14.5	12.4	13.4
Cys-Cys	3.3	3.3	4.2	3.6
Glutenin II				
Lys	4.6	4.1	5.2	5.1
Pro	9.4	10.6	10.0	9.6
Cys-Cys	2.0	2.1	1.9	2.4
Glutenin III				
Lys	4.2	4.2	4.4	4.5
Pro	10.5	10.7	9.8	9.1
Cys-Cys	2.5	3.5	3.3	3.4

contains more cystine (3.2 against 2.1 p. 100), methionine (2.6 against 1.8 p. 100), leucine (8.1 against 5.6 p. 100).

The amino acid compositions of the same fractions show some differences between wheat varieties (Table 5).

The data on amino acid analysis of water-soluble and insoluble gliadins and glutenins are of interest (Table 6). There is no doubt that division into sub-

TABLE 6

Amino Acid Composition of Water-Soluble and Insoluble Subfractions

Amino acids	Gliadin		Glutenin I		Glutenin II		Glutenin III	
	H_2O-soluble	ethanol-soluble	soluble	insoluble	soluble	insoluble	soluble	insoluble
Lys	0.83 ± 0.07	0.92 ± 0.13	6.0 ± 0.34	0.57 ± 0.11	4.5 ± 0.31	5.3 ± 0.33	4.6 ± 0.10	3.0 ± 0.27
His	1.8 ± 0.21	1.6 ± 0.12	1.8 ± 0.21	1.7 ± 0.07	1.5 ± 0.07	2.6 ± 0.14	2.0 ± 0.05	2.1 ± 0.10
Arg	1.6 ± 0.14	1.7 ± 0.07	3.4 ± 0.41	1.8 ± 0.21	3.0 ± 0.24	5.5 ± 0.30	4.2 ± 0.19	4.0 ± 0.11
Thr	1.9 ± 0.09	2.1 ± 0.19	5.0 ± 0.18	2.2 ± 0.07	3.6 ± 0.07	3.0 ± 0.22	3.7 ± 0.11	3.0 ± 0.14
Asp	1.9 ± 0.07	2.4 ± 0.14	9.4 ± 0.21	1.8 ± 0.06	5.1 ± 0.57	7.3 ± 0.65	6.3 ± 0.13	4.2 ± 0.46
Ser	4.0 ± 0.17	4.2 ± 0.15	4.0 ± 0.26	4.5 ± 0.06	4.0 ± 0.24	5.6 ± 0.25	5.3 ± 0.47	4.9 ± 0.16
Glu	42.0 ± 0.65	37.1 ± 1.12	14.6 ± 0.40	40.4 ± 0.31	32.8 ± 1.70	26.5 ± 0.83	24.3 ± 0.83	28.8 ± 1.38
Pro	14.5 ± 0.31	16.4 ± 0.99	7.0 ± 0.57	14.3 ± 0.65	10.2 ± 0.75	9.3 ± 0.80	9.5 ± 0.21	13.5 ± 2.36
Gly	2.7 ± 0.25	2.8 ± 0.31	6.2 ± 0.43	2.2 ± 0.19	9.0 ± 0.36	5.7 ± 0.22	5.6 ± 0.29	5.0 ± 0.41
Ala	2.2 ± 0.03	2.6 ± 0.09	8.0 ± 0.49	2.5 ± 0.15	4.8 ± 0.15	3.5 ± 0.19	4.2 ± 0.10	3.3 ± 0.16
Cys-Cys. . . .	3.3 ± 0.19	3.0 ± 0.19	3.9 ± 0.69	3.6 ± 0.25	1.5 ± 0.18	3.2 ± 0.51	3.0 ± 0.26	4.0 ± 0.07
Val	3.8 ± 0.14	3.5 ± 0.20	7.3 ± 0.41	3.9 ± 0.11	3.2 ± 0.25	6.0 ± 0.36	4.6 ± 0.10	4.5 ± 0.33
Met	1.8 ± 0.15	1.6 ± 0.41	1.6 ± 0.46	2.5 ± 0.15	1.8 ± 0.24	1.9 ± 0.20	2.7 ± 0.12	2.2 ± 0.13
Ile.	3.9 ± 0.09	4.1 ± 0.22	4.3 ± 0.15	4.2 ± 0.06	2.4 ± 0.20	3.2 ± 0.24	3.5 ± 0.09	3.6 ± 0.56
Leu	6.6 ± 0.28	7.1 ± 0.27	7.5 ± 0.10	7.1 ± 0.10	5.4 ± 0.26	6.2 ± 0.12	8.2 ± 0.27	7.7 ± 0.52
Tyr	2.4 ± 0.14	3.1 ± 0.39	2.5 ± 0.31	1.9 ± 0.10	4.4 ± 0.43	traces	3.9 ± 0.21	4.6 ± 0.68
Phe	4.9 ± 0.26	5.9 ± 0.17	3.6 ± 0.28	5.3 ± 0.18	2.5 ± 0.20	4.7 ± 0.17	4.2 ± 0.14	4.4 ± 0.24

Each value represents the mean ± SE for four varieties.

fractions according to water solubility is caused by different chemical properties of proteins. Let's consider glutenins first.

The soluble glutenin I subfraction which is a very small part of flour protein (0.4 p. 100) has several times more lysine, arginine, aspartic acid, threonine, alanine, valine but much less glutamic acid, proline, methionine than insoluble subfraction. The first resembles albumin and globulin whereas the second relates to gliadin. The insoluble protein differs from gliadin in that the first contains more methionine (2.5 against 1.6-1.8 p. 100) and possible cystine (3.6 against 3.0-3.3 p. 100). The content of cystine is high (3.9 p. 100) in soluble part of glutenin I. Fractions with amino acid compositions alike soluble and insoluble glutenin I were isolated by other methods (4).

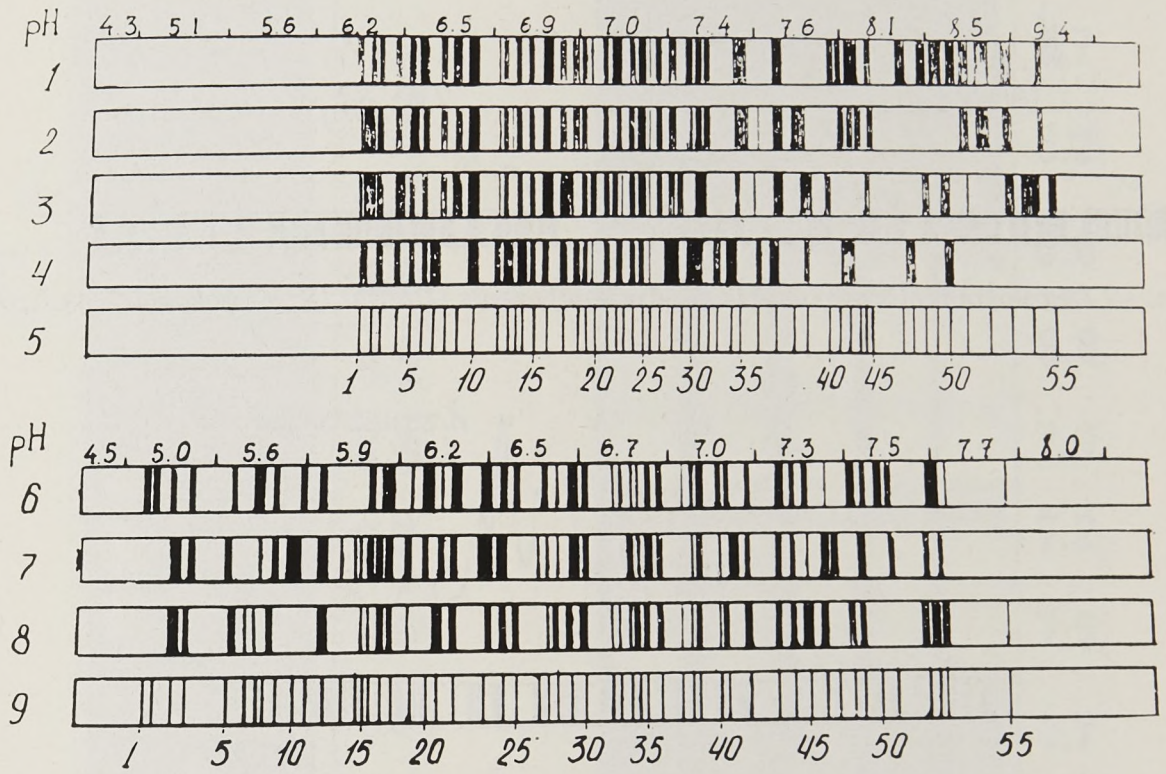

FIG. 1. — *The scheme of gliadin component distribution in gel after isoelectric focusing; above: water-soluble subfraction: below; ethanol-soluble subfraction.*
1. *Bezostaya 1; 2,6 Krasnodarskaya 46; 3.7 Saratovskaya 29; 3,4 Voskhod; 5,9 total spectrum.*

Soluble and insoluble glutenin II subfractions differ from each other in quantity of histidine, arginine, glycine, valine and phenylalanine. It was a surprise that the insoluble subfraction contained traces of tyrosine only. It was typical of all flour varieties.

The soluble glutenin III contains more lysine and aspartic acid than insoluble protein, the latter has the highest level of cystine (4 p. 100). Probably, this protein group resembles high-molecular weight, highly polar polypeptides.

The water-soluble and ethanol-soluble gliadins are very much alike in amino acid composition. More or less significant differences can be noted in aspratic and glutamic acids, tyrosine and phenylalanine. However, isoelectric focusing demonstrates some differences (Fig. 1). The components of soluble proteins run towards the basic isoelectric points of pH while that of insoluble — towards the acidic ones. Such behaviour may be caused by different level of amidation of glutamic and aspartic acids. In addition, we may think that water-soluble

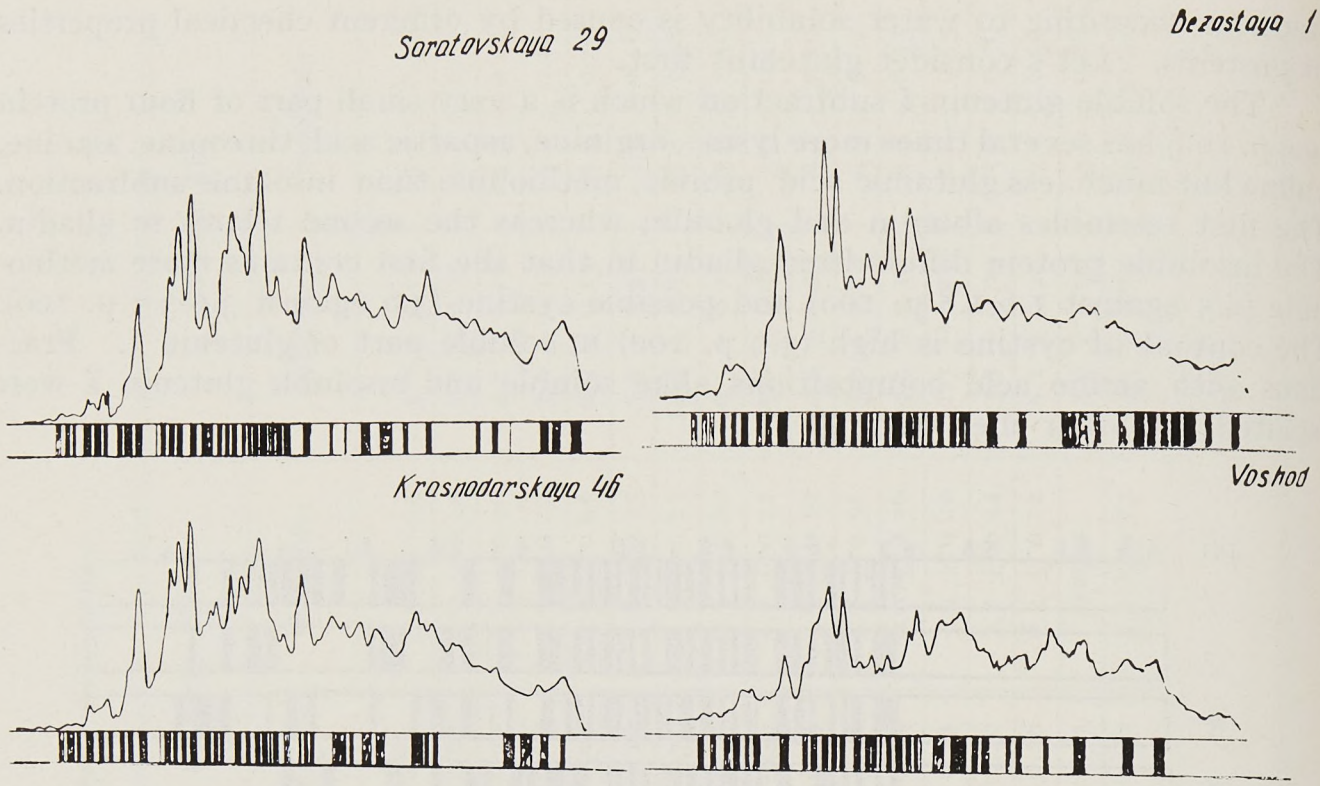

FIG. 2. — *Densitogram of water-soluble gliadin components (Digital Densitorol DMU-33 C, Toyo).*

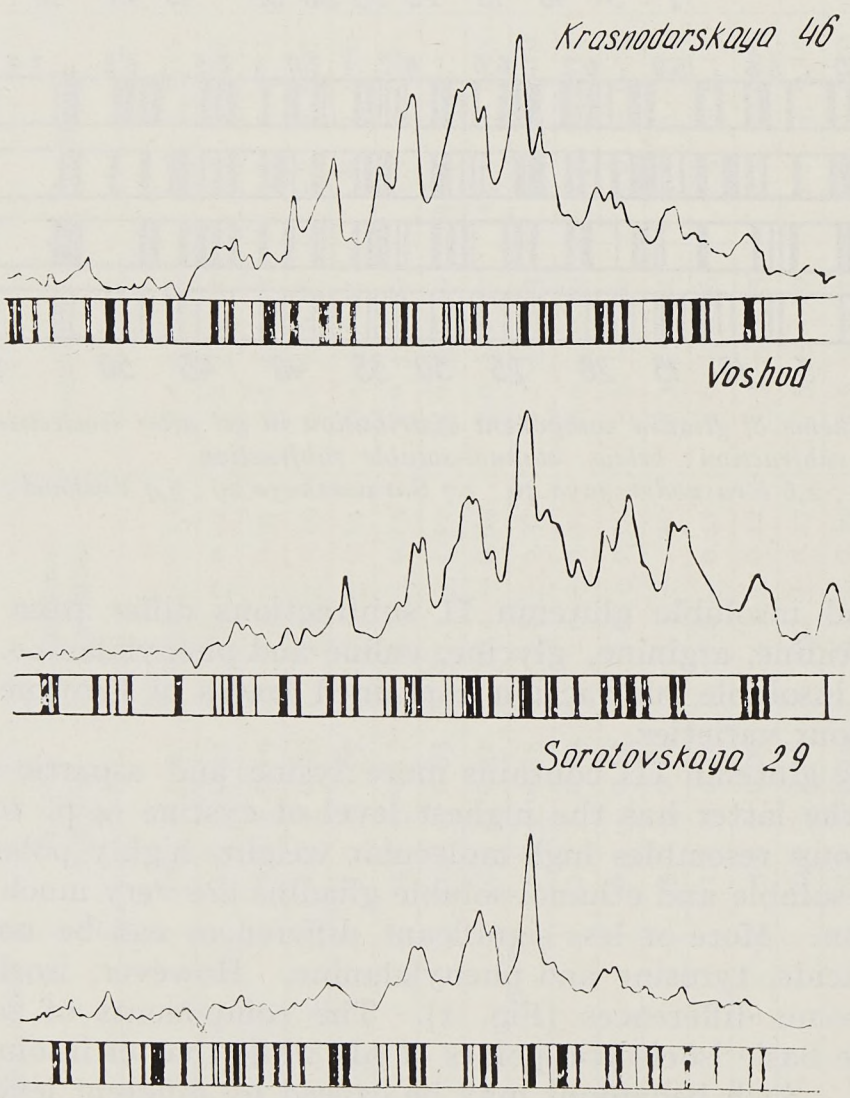

FIG. 3. — *Densitogram of ethanol-soluble gliadin components (Digital Densitorol DMU-33 C, Toyo).*

gliadins are presented by low-molecular weight subunits whereas insoluble ones by high-molecular weight or associated groups of molecules.

The significant background which takes place in spaces between the components of water-soluble and insoluble gliadins attracts much attention. Unsufficient resolution of peaks on densitograms and rising of base line confirms it (Figs. 2, 3). At the same time the spectrum of non-dialysed gliadin looks clear (Figs.

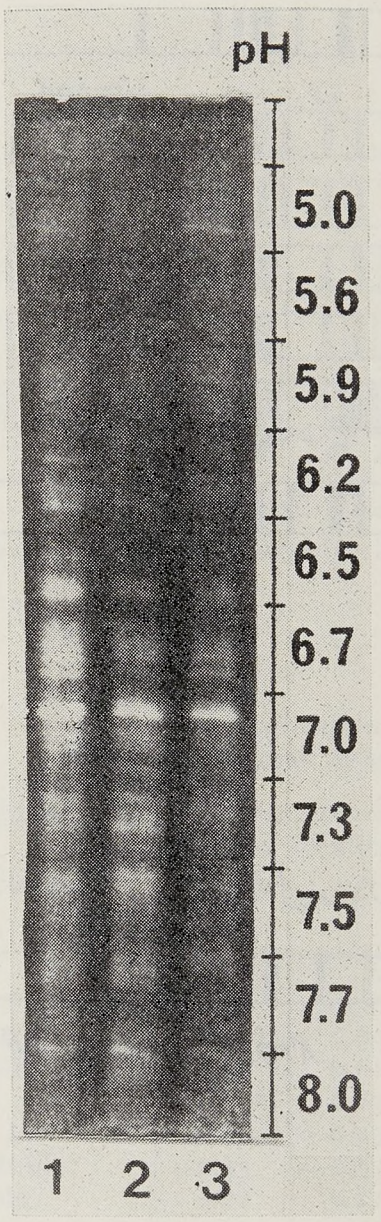

FIG. 4.

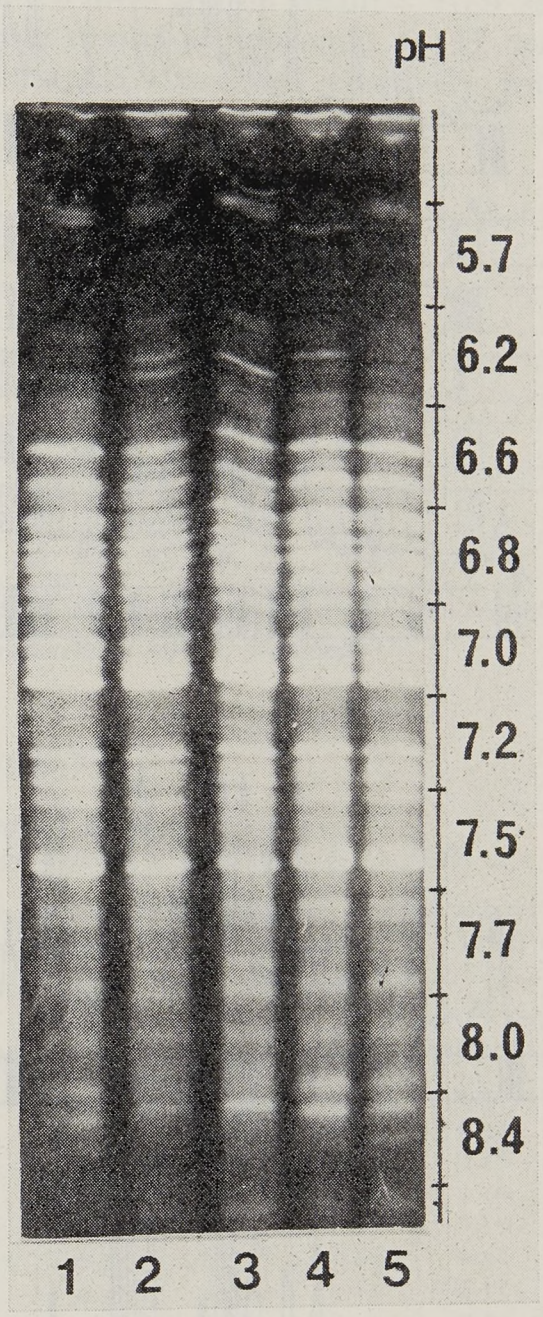

FIG. 5.

FIG. 4. — *The spectrum of gliadin water-insoluble (ethanol-soluble) components after isoelectric focusing.*

1. *Saratovskaya 29;*
2. *Voskhod;*
3. *Krasnodarskaya 46.*

FIG. 5. — *The spectrum of total gliadin components (without dialysis) in gel after isoelectric focusing.*

1, 5. *Bezostaya 1;*
2. *Krasnodarskaya 46;*
3. *Voskhod;*
4. *Saratovskaya 29.*

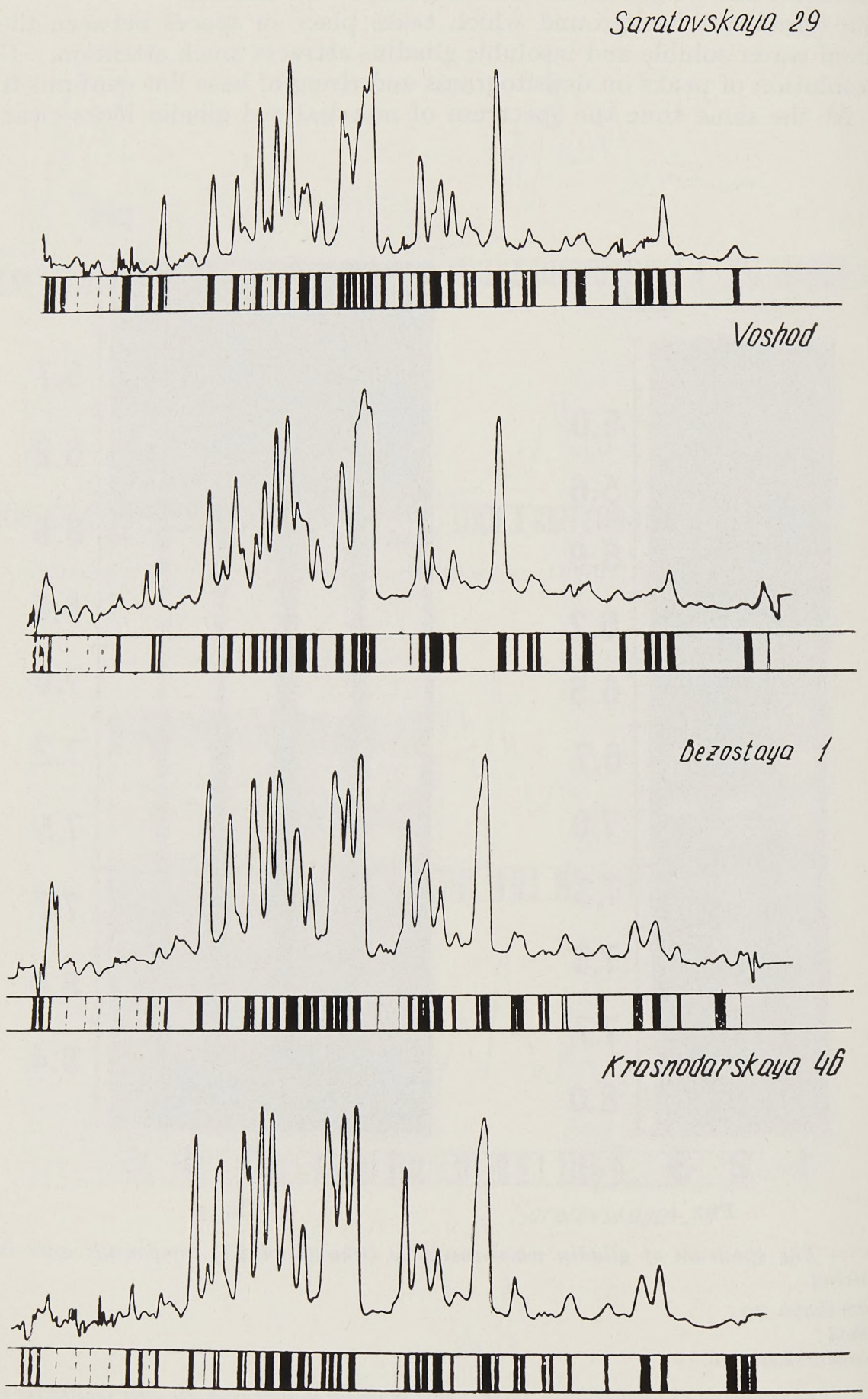

FIG. 6. — *Densitogram of total gliadin components (without dialysis) (Digital Densitorol DMU-33 C, Toyo)*

4, 5, 6). The reasons of these differences are still not known to us. The total spectrum for 4 wheat varieties counts 55 protein components in each gliadin subfraction and that of total gliadin 65 (Fig. 7). It may be assumed that the individual bands of total gliadin are presented not by single protein only but include several components belonging to the water-soluble and ethanol-soluble gliadins. Varietal differences on number, position and expressiveness of bands relating to genotypical specifity should be mentioned.

Water-soluble glutenin I, II correlated with gluten quality, flour strength, dough resistance negatively but water-insoluble glutenin I, II positively (Table 7). The amount of total glutenin III correlated positively with flour

FIG. 7. — *The scheme of total gliadin component distribution in gel after isoelectric focusing. 1. Bezostaya 1 ; 2. Krasnodarskaya 46 ; 3. Saratovskaya 29 ; 4. Voskhod.*

TABLE 7

Correlation Coefficient (r) Between Glutenin subfractions and technological properties of Wheat flour (n = 8)

Fractions	Gluten quality		Flour strengh		Resistance		Loaf volume	
	N	F	N	F	N	F	N	F
Glutenin I	—0.36	0.30	0.0	0.0	0.23	0.53	0.34	0.13
H_2O-soluble. . .	—0.71*	—0.69	—0.64	—0.45	—0.70*	—0.26	0.54	0.43
Insoluble	0.56	0.61	0.18	0.26	0.45	0.66	0.17	0.0
Ins /sol	0.79*	0.84*	0.52	0.49	0.70*	0.49	0.19	0.38
Glutenin II	—0.39	—0.29	—0.35	—0.11	—0.38	—0.17	0.10	—0.16
H_2O-soluble. . .	—0.35	—0.53	—0.77*	—0.70	—0.77*	—0.89*	0.52	0.39
Insoluble	—0.21	0.21	0.0	0.10	0.0	0.10	—0.12	—0.26
Ins /sol	0.10	0.05	0.38	0.41	0.26	0.42	—0.33	—0.40
Glutenin III . . .	0.47	0.60	0.68	0.81*	0.15	0.31	—0.53	—0.75*
H_2O-soluble. . .	0.39	0.45	0.59	0.64	0.57	0.62	—0.66	—0.78*
Insoluble	0.26	0.36	0.35	0.46	0.23	—0.14	—0.13	—0.28
Sol /insol	—0.15	—0.17	—0.16	—0.18	0.34	0.33	—0.12	—0.12
Sol (GII + GIII) A	—0.55	—0.77*	—0.79*	—0.73*	—0.82*	—0.73*	0.58	0.52
Ins (GII + GIII) B	0.31	0.30	0.18	0.30	0.44	0.58	0.0	0.33
B /A	0.52	0.69	0.68	0.68	0.78*	0.82*	—0.41	—0.54
Ins GII /sol GIII .	0.72*	0.84*	0.73*	0.49	0.89*	0.49	—0.31	—0.38

*Correlation coefficient is significant, p. < 0.05 N, (p. 100 of total N). F (p. 100 of total flour).

strength but negatively with loaf volume. A closer relationship was observed between qualities and soluble part of glutenin III than with insoluble one. Probably, insoluble glutenins I, II and soluble subfraction of glutenin III are high-molecular weight proteins which are able to form disulphide bonds (6, 9, 10). A higher correlation between baking qualities and proportion of insoluble to soluble glutenins I, II and proportion of insoluble glutenin I to soluble glutenin II was found than correlation with absolute amounts of these proteins. It seems that rheological properties of gluten and dough much more depend upon ratios of protein subfractions than upon their absolute content. However, the correlation analysis should be carried out using greater numbers of flour varieties to get conclusions convincing enough.

Reçu pour publication en octobre 1980.

Résumé

Étude des gluténines de la farine de blé par les méthodes Landry-Moureaux modifiées et des gliadines par électrofocalisation

Nous avons extrait les gluténines (Glu) de farine de 12 échantillons de blés par la méthode Landry-Moureaux. Ensuite, les Glu I, II, III et gliadines (Gli) sont fractionnées en sous-fractions solubles dans l'eau (S) et insolubles dans l'eau (Ins) par dialyse. Ces fractions représentent par rapport à l'azote total: Glu I : 3,8-6,0 (dont S 0,4, Ins 3,4-5,6); Glu II : 1,5-2,4 (dont S 1,0-1,6 Ins 0,4-0,8); Glu III : 19,6-26,9 (dont S 17,0-22,7, Ins 2,2-3,6); Gli : 43,7-51,7 (dont S 14,4-23,3, Ins 28,4-30,7).

L'analyse des acides aminés montre des différences importantes qui indiquent que les sous-fractions de la gluténine représentent des groupes de protéines différents. Glu I est du type Gliadine, mais sa sous-fraction soluble est du type albumine (6 p. 100 de lysine). Glu II et Glu III ont respectivement 4,8 et 4,3 p. 100 de lysine, 30,7 et 24,8 p. 100 d'acide glutamique, 9,9 et 10,0 p. 100 de proline. Ins Glu II présente des traces de tyrosine. Les différences dans la composition en acides aminés entre les gliadines S et Ins n'est pas significative mais leur comportement en électrofocalisation est différent. Les constituants S Gli migrent vers les points isoélectriques basiques et les Ins Gli vers les points isoélectriques acides. Les variétés diffèrent par le nombre et la position des bandes. Le nombre total des bandes pour 4 variétés de blé est le suivant :

S-Gli 55, Ins-Gli 55, S + Ins 65. L'analyse de 8 échantillons de farine de blé montre une corrélation positive entre Glu III et la force de la farine, et une corrélation négative entre S Glu I, S Glu II, leur somme et la force de la farine. La corrélation positive est meilleure lorsque l'on compare la force de la farine à des rapports tels que Ins Glu I /S Glu I, Ins Glu I /S Glu II.

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SESSION V

Gluten

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Correlation between chemical structure and rheological properties of gluten

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Summary

Generally it can be stated that two main groups of factors may influence the rheological properties of the gluten:

— the quality and quantity of the protein fractions in the gluten complex (amino acid composition, molecular weight and shape, etc...),

— the interaction between the different protein components of the gluten complex (disulphide bonds, hydrogen bonds, hydrophobic interactions, etc...).

In the framework of the research work in our laboratory the influence of the following factors was investigated:

— amino acid composition;

— ratio of low and high molecular weight fractions;

— interactions between protein components.

It was stated that the cystine content, degree of amidation and content of hydrophobic side chain amino acids have a significant effect on the rheological properties.

The lower molecular weight fractions determined by gel chromatography, gel electrophoresis or peptization decrease the rheological quality of the gluten complex.

The quantity and distribution of disulphide bonds play a very important role in the deter mination of the rheological properties. The distribution of disulphide bonds may be changed by reduction and reoxydation of the gluten complex.

Investigation of chemically modified gluten showed that in the forming of intermolecular hydrogen bonds the amido and primary amino groups play the main role.

Key words : *gluten — rhéological properties — interaction.*

I. — Introduction

The prominent position of wheat resp. wheat flour among the raw materials of baking industry is strongly connected with his unique proteins. Amongst the cereals, only the bread wheats — and to lesser extent the triticale — possess storage proteins which interact with water to yield doughs having the ne-

cessary cohesiveness and elasticity for making leavened bread. The reasons for this, and the structure of gluten and its protein constituents have intrigued cereal chemists since the days of Osborne. It is evident that the factors playing role in the determining the rheological properties of gluten are of complex character. On the basis of theoretical considerations and also from all the studies which have been carried out it would appear that the following two groups of factors are the most important:

— the quantity and quality (solubility, aminoacid composition, molecular weight distribution etc.) of the protein components of the gluten complex;

— the interactions (disulphide bonds, hydrogen bonds, hydrophobic interactions, electrostatie interactions etc.) between the protein fractions.

In the framework of the research work of our laboratory the correlation between the chemical structure and the rheological properties of gluten complex was investigated. The effect of aminoacid composition, the molecular weight distribution of proteins and, as most important, the interactions of proteins were studied. The results of this investigations are discussed in this paper and compared with the conclusions of other authors.

II. — Materials and methods

Glutens were prepared from different varieties of wheats resp. wheat flours (Bezostaya, Fertödi 279, San Pastore, Kavkaz, Jubelejnaja, MV4) by washing and additional purification. The method was described earlier (LÁSZTITY, 1960, 1967).

The rheological properties of the gluten were characterized by a three element model (combined from the Maxwell and Hooke model; H/H-N). The relaxation time and the relaxation constant were calculated. The stress relaxation was measured by a modified neolaborograph. The details of the measurement and calculations were published in an earlier paper (LÁSZTITY, 1967).

The aminoacid composition of the gluten proteins was determined after hydrolysis with hydrochloric acid by an automatic aminoacid analyzer (AAA 881 Mikrotechna, Praha).

The separation of the protein fractions of the gluten complex was made by gel chromatography and peptization by acetic acid solutions. The details of the methods were described also earlier (LÁSZTITY 1969 *a*, 1970).

Chemically modified glutens (deamidated-, acetylated-, esterified-, glutens, N-ethyl-maleimid derivatives) were prepared with the methods described by LÁSZTITY (1969), BECKWITH *et al.* (1963), BARNAY *et al.* (1965), HOLME and BRIGGS (1959). For splitting of disulphide bonds and reoxydation of them the method of BECKWITH *et al.* (1965, 1966) was used.

III. — Results and discussion

3.1. — *Aminoacid composition*

The aminoacid composition of the glutens prepared from different wheats shows smaller differences which may be in many cases statistically significant. Nevertheless the aminoacid composition of the gluten is relatively stable and may be characterised as follow:

— high glutamic acid content;
— relatively high proline content;
— low amount of basic aminoacids (lysine, arginine, histidine);
— high degree of amidation nearly equivalent with the aspartic and glutamic acid content;
— remarkable cystein resp. cystine content.

The mathematical — statistical evaluation of the results of the gross aminoacid composition and the rheological characteristics in most cases does not give significant correlation. The total disulphide bonds (cystine) content shows a significant correlation with the rheological properties characterised by a linear correlation coefficient variing between 0.3-0.6. A strong positive correlation between the cystine content and wheat flour baking value was reported earlier by WÖSTMANN (1950). The lower degree of correlation may be explained by the hypothesis, that not only the absolute number of disulphide bonds but also the distribution of them is important from viewpoint of the rheological properties. The problem of the disulphide bonds will be discussed later in the chapter 3.3. of this paper.

The correlation between the degree of amidation (calculated on the basis of aspartic and glutamic acid content resp. ammonia content) and rheological characteristics is shown in the Figure 1.

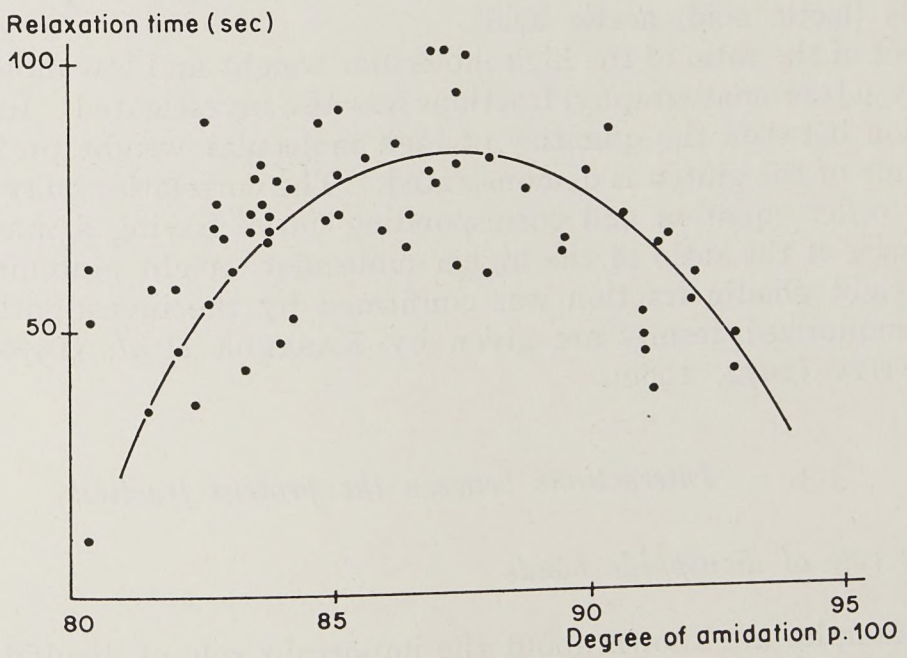


FIG. 1. — *Correlation between the degree of amidation and the relaxation time of gluten.*

According to the calculation an optimal degree of amidation exists. The observation partly may be explained by the work of BRIGGS and HOLME (1959), CUNNINGHAM *et al.* (1955), BECKWITH *et al.* (1963). They postulated that an increase of the number of the amide groups increase the possibility of the formation of secondary bonds in the gluten structure which improve the rheological properties of the gluten. However this hypothesis does not explain the negative effect of highest degrees of amidation.

An interesting correlation was found between the amount of amino acids with hydrophobic side chain (leucine, isoleucine, proline) and the relaxation time. The correlation may be described by a second order equation.

On the basis of the results mentioned above a new " quality number " was proposed for the preliminary evaluation of the wheat baking value from the data of the amino acid composition of the gluten.

$$(1)\quad QN = 10\ C + 0{,}15\ A - K_A + 0{,}4\ H - K_H$$

where C = cystine content (p. 100)
A = degree of amidation (p. 100)
H = proline, leucine and isoleucine content
$K_A = 87 - A$ (absolute value)
$K_H = 22{,}5 - H$ (absolute value)

3.2. — *Protein fractions distribution*

The easily dispersible (0.05 N acetic acid solution) and not dispersible fraction of the gluten complex was measured and the correlation between the quantities of protein fractions and the relaxation time were calculated statistically. The results are shown in the Figures 2. and 3. On the basis of the results it can be stated that the amount of easily dispersible components is in negative correlation with the rheological quality. The increase of the quantity of non peptisable fractions has an improving effect. This observations are in good agreement with the statements of many authors and with some methods of determination of wheat baking value based on the swelling or dispersibility of gluten proteins in dilute organic acids (lactic acid, acetic acid).

The effect of the ratio of the high molecular weight and low molecular weight (separated by gel chromatography) fractions was also investigated. In the Figure 4. the correlation between the quantity of high molecular weight proteins and the relaxation time of the gluten is demonstrated. The correlation may be expressed by a second order equation and corresponding curve having a maximum value. The importance of the ratio of the higher molecular weight glutenin to the lower molecular weight gliadin fraction was confirmed by the investigations of many authors. Summarized results are given by KASARDA *et al.* (1976), SIMMONDS (1979), LÁSZTITY (1972, 1980).

3.3. — *Interactions between the protein fractions*

3.31. — *The role of disulphide bonds*

Many examples are known about the important role of disulfide bonds concerning the protein structure and its mechanical properties. The gluten proteins

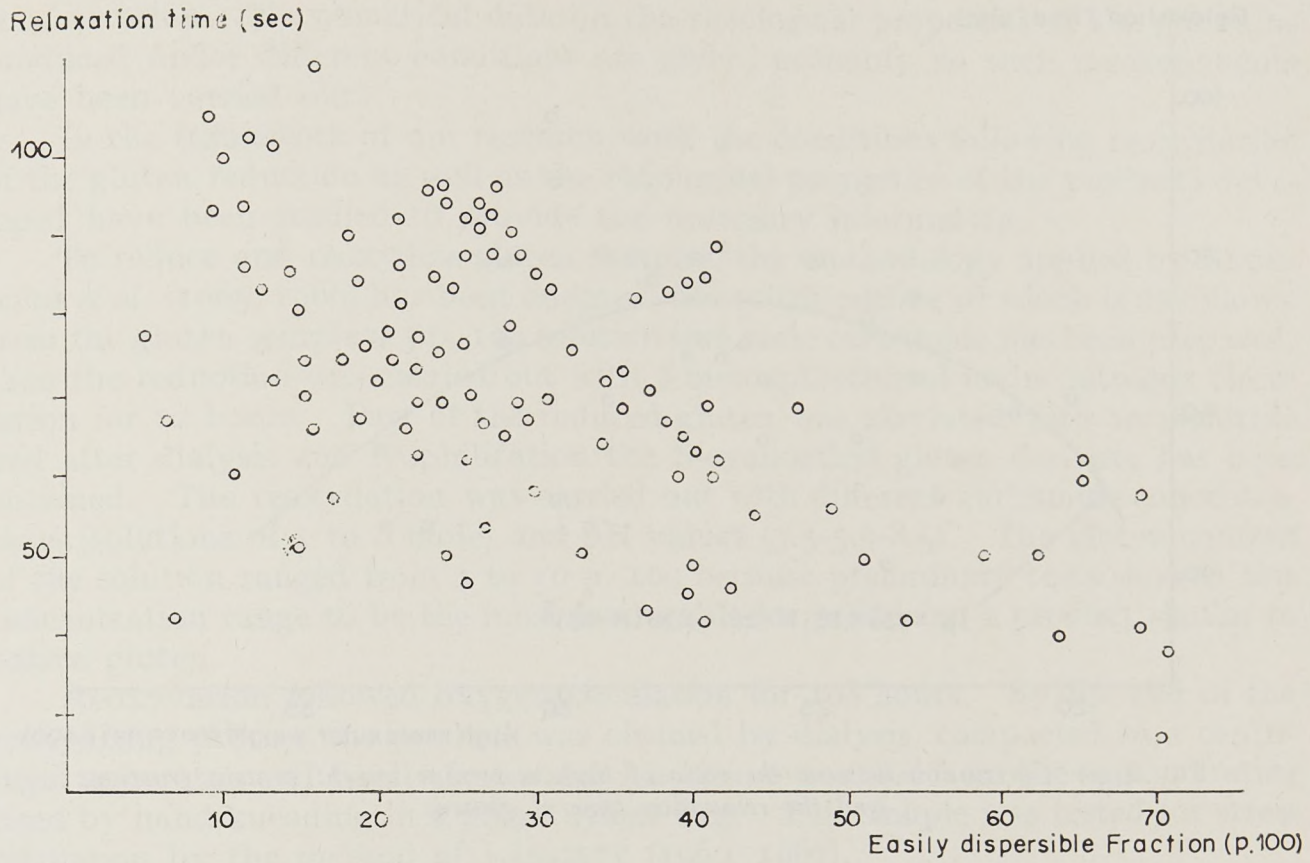


FIG. 2. — *Correlation between the quantity of easily dispersible protein fractions and the relaxation time of glutens.*

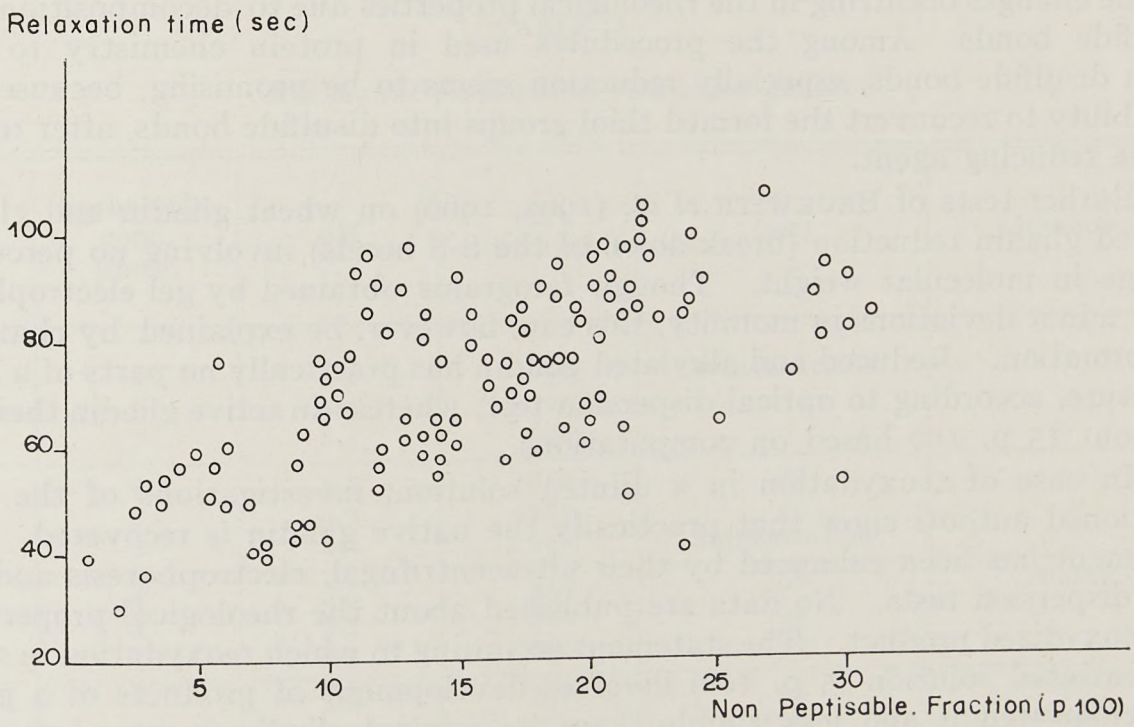


FIG. 3. — *Correlation between the quantity of non peptisable protein fractions and the relaxation time of glutens.*

contain relatively small quantities of cystine and cysteine, respectively (in average) 2 to 3 p. 100. Primarily their importance was called upon in relation with problems of flour improvers. In consequence, a very intensive research work was started in this domain, especially in the last two decades. Nevertheless, there

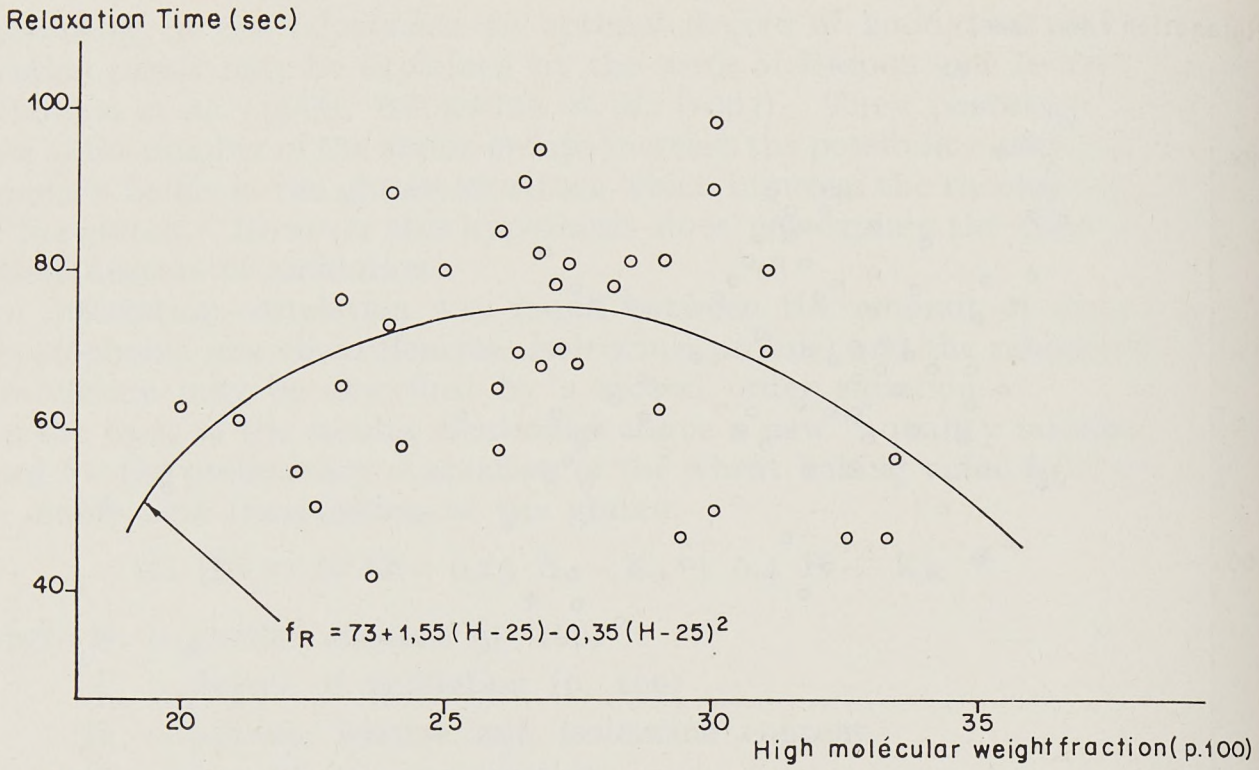


FIG. 4. — *Correlation between the ratio of high molecular weight protein fractions and the relaxation time of glutens.*

are still many unsolved problems. To clear up the role of disulfide bonds involves further manysided investigations. Some useful data may be obtained by studying the changes occurring in the rheological properties due to decomposition of the disulfide bonds. Among the procedures used in protein chemistry to break down disulfide bonds, especially reduction seems to be promising, because of the possibility to reconvert the formed thiol groups into disulfide bonds, after removal of the reducing agent.

Earlier tests of BECKWITH *et al.* (1965, 1966) on wheat gliadin and glutenin showed gliadin reduction (break-down of the S-S bonds) involving no perceptible change in molecular weight. Though ferograms obtained by gel electrophoresis show minor deviations in mobility, this can, however, be explained by changes in conformation. Reduced and alkylated gliadin has practically no parts of a helical structure, according to optical dispersion test, whereas in active gliadin their ratio is about 15 p. 100 based on computations.

In case of reoxydation in a diluted solution, investigations of the above mentioned authors show that practically the native gliadin is recovered. Their statement has been enhanced by their ultracentrifugal, electrophoresis and rotatory dispersion tests. No data are published about the rheological properties of the reoxydized product. The statement according to which reoxydation in a more concentrated solution (5 p. 100) involves development of products of a greater molecular weight and less soluble than the original gliadin is very interesting. It is supposed that intermolecular disulfide bonds are forming in this case.

The decomposition of the disulfide bonds of glutenin by reduction resulted an disintegration of the large gluten component molecules. In case of reoxydation in solutions of higher concentration, a product with properties very near to those of native glutenin may be obtained. According to information of the authors, physical properties of the product formed in course of reoxydation are much influenced by the reoxydation conditions, primarily by pH and the carbamid

concentration. No numerical data on the rheological properties of the glutenins produced under different conditions are given, probably no such measurements have been carried out.

In the framework of our research work the conditions following reoxydation of the gluten reduction as well as the rheological properties of the products developed have been studied to provide the necessary information.

To reduce and reoxydize gluten samples, the methodology applied by BECKWITH *et al.* (1965, 1966) has been adopted, the rough outline of which is as follows: from the gluten sample a 5 p. 100 solution in 6 mole carbamide has been prepared, then the reduction was carried out with β-mercaptoethanol under nitrogen circulation for 12 hours. Part of the reduced gluten was alkylated with acrylonitrile and after dialysis and lyophilization the S-cyanoethyl gluten derivate has been obtained. The reoxydation was carried out with different carbamide concentrations (solutions of 1 to 8 mole) and pH values (3.5-5.5-8.5). The gluten content of the solution ranged from 1 to 10 p. 100 because preliminary tests showed this concentration range to be the most favourable for producing a product similar to native gluten.

Reoxydation followed oxygen circulation for 168 hours. By the end of the reoxydizing process the product was cleaned by dialysis, compacted in a centrifugal apparatus and finally free water has been evacuated from the agglomerating mass by hand kneading in a polyethylene bag. This sample was tested for stress relaxation by the method of LÁSZTITY (1960, 1967).

Table 1 recapitulates the rheological properties of reoxydized glutens, produced under different conditions.

TABLE 1

Rheological properties of reoxydized glutens

No.	Carbamide conc. (mole)	pH	τ_h din/cm²	t_r (sec)	Remarks
1 . . .	1	3.5	150	immensurable	—
2 . . .	1	5.5	220		
3 . . .	1	8.5	350		
4 . . .	2	3.5	240	30	—
5 . . .	2	5.5	280	42	
6 . . .	2	8.5	1 200	immensurable	
7 . . .	4	3.5	540	68	elastic
8 . . .	4	5.5	770	92	
9 . . .	4	8.5	1 800	150	
10 . . .	6	3.5	300	42	slightly elastic
11 . . .	6	5.5	270	29	
12 . . .	6	8.5	1 900	180	
13 . . .	8	3.5	320	35	—
14 . . .	8	5.5	2 700	immensurable	nonelastic
15 . . .	8	8.5	2 800	immensurable	nonelastic
16 . . .	native gluten	(control)	590	83	gluten of average elasticity and extensibility

Data in Table 1 show that the rheological properties of the developing products are highly influenced by the reoxydation conditions. Products best approaching the properties of native gluten may be produced with a carbamide solution of 4 mole at pH 5.5 and with a carbamide solution of 6 mole at pH 3.5. In general, with a low carbamide concentration a cohesive product without elastic properties is obtained, whereas in case of a 8 mole carbamide concentration, the reoxydized gluten is tougher but has no appropriate elasticity. In alcaline media (pH 8.5) no product with properties similar to native gluten could be obtained in either carbamide concentration. It has to be noted that reoxydation was essentially more rapid in an alcaline medium. In general, the reoxydized product yielded a

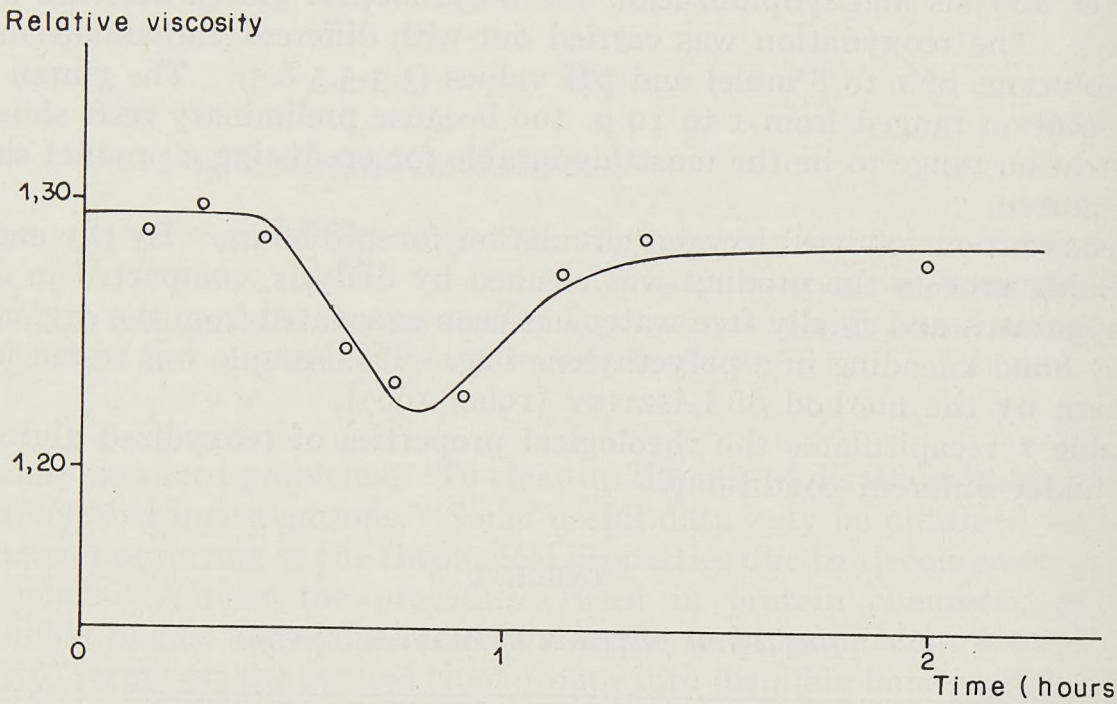


FIG. 5. — *Changes of the viscosity of reoxydised gluten solution during splitting of S-S bonds by performic acid (Product A; reoxydation in 1M Carbamide, pH' = 3,5).*

practically inelastic, cohesive mass when mixed with water. Its properties could be compared for the most part with gluten extensively denaturated by heat.

The present study of the rheological properties shows distinctly the important role of disulfide bonds in the development of the physical properties of gluten. At the same time it is distinctly apparent that the absolute number of disulfide bonds alone does not determine unambiguously the above properties. In this respect also the position of the disulfide bonds is essential.

The influence of pH and the carbamide concentration on the properties of the reoxydized products can be explained as follows: depending on the pH and the carbamide concentration, changes may occur in the conformation of the peptide chains, in the steric position of the individual groups, their dissociation conditions, their reactivity, defining the type and place of the forming disulfide bonds. The effect of carbamide, of the pH, and the ion concentration on the protein conformation is generally known.

The forming disulfide bonds might be either intramolecular or intermolecular bonds. The ratio of inter- and intramolecular disulfide bonds is supposed to be very important in the development of the rheological properties. To check this supposition, the change in viscosity of the solution in course of both performic

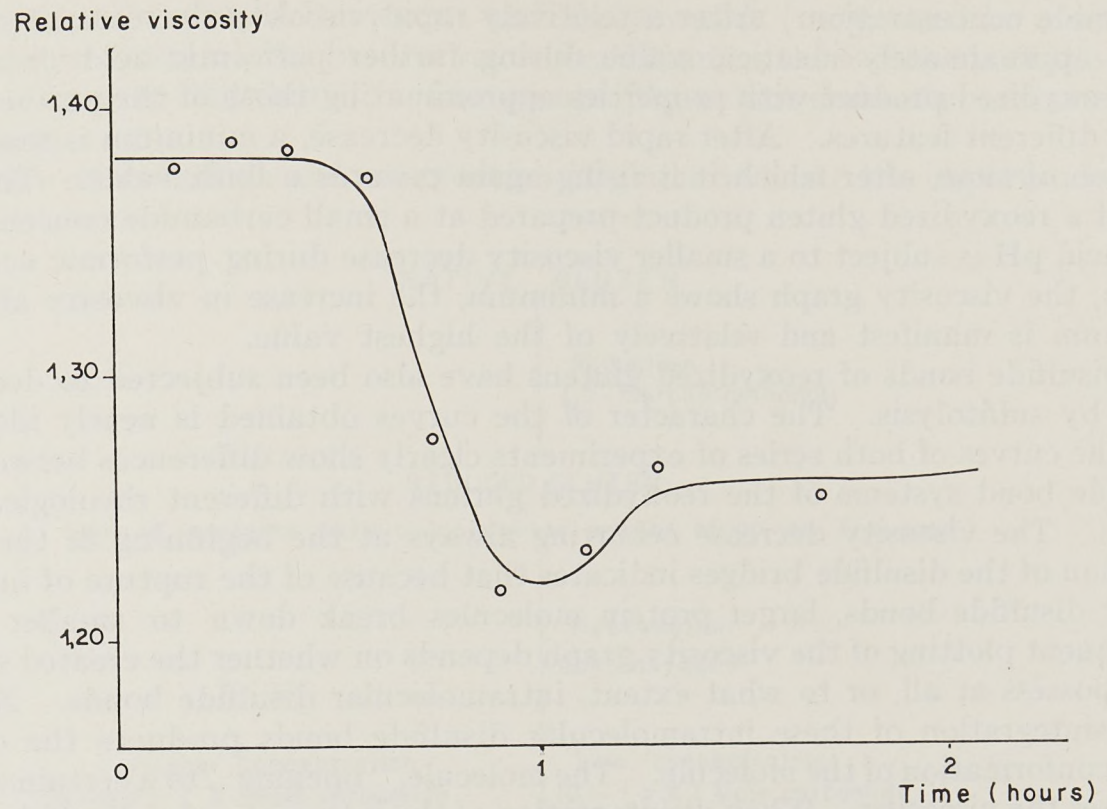


FIG. 6. — *Changes of the viscosity of reoxydised gluten solution during splitting of S-S bonds by performic acid (Product B : reoxydation in 3 M Carbamide, pH = 5,5).*

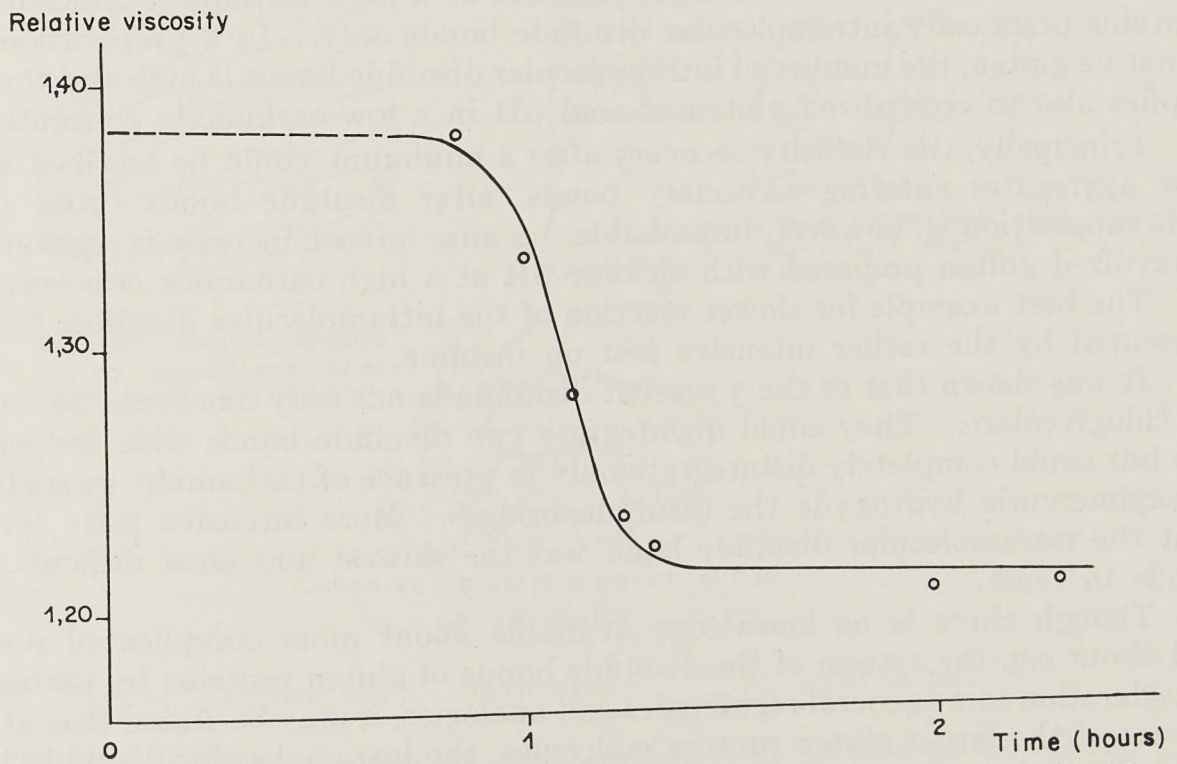


FIG. 7. — *Changes of the viscosity of reoxydised gluten solution during disrupting of S-S bonds by performic acid (Product C : reoxydation in 8 M Carbamide, pH = 8,5).*

acid decomposition and sulfitolysis of reoxydized glutens obtained in different ways has been investigated. Characteristic curves are shown in Figure 5, 6 and 7.

It can be distinctly seen that the viscosity graphs versus time are different for reoxydized gluten products of different rheological properties. The simplest graph has been obtained for a reoxydized gluten in alcaline medium at a higher

carbamide concentration. After a relatively rapid viscosity decrease, it remains at an approximately identical value during further performic acid oxydation. The reoxydized product with properties approximating those of the native gluten shows different features. After rapid viscosity decrease, a minimum is reached in function of time, after which it is rising again towards a limit value. The solution of a reoxydized gluten product prepared at a small carbamide concentration with acid pH is subject to a smaller viscosity decrease during performic acid oxydation, the viscosity graph shows a minimum, the increase in viscosity after the minimum is manifest and relatively of the highest value.

Disulfide bonds of reoxydized glutens have also been subjected to decomposition by sulfitolysis. The character of the curves obtained is nearly identical.

The curves of both series of experiments clearly show differences between the disulfide bond systems of the reoxydized glutens with different rheological properties. The viscosity decrease occurring always at the beginning of the disintegration of the disulfide bridges indicates that because of the rupture of intermolecular disulfide bonds, larger protein molecules break down to smaller units. Subsequent plotting of the viscosity graph depends on whether the created smaller units possess at all, or to what extent, intramolecular disulfide bonds. Namely the disintegration of these intramolecular disulfide bonds produces the change of the conformation of the molecule. The molecule, " opening .. to a certain extent, increases the viscosity. When disintegration of the intramolecular disulfide bonds comes to an end, both the molecule conformation and the viscosity show constant values. Studying the curves from this point of view, it is seen that the reoxydized gluten prepared in an alcaline medium at a high carbamide concentration contains practically intramolecular disulfide bonds only. In a preparation close to native gluten, the number of intramolecular disulfide bonds is high and the same applies also to reoxydized gluten of acid pH in a low carbamide concentration.

Principally, the viscosity recovery after a minimum could be ascribed also to new aggregates entering secondary bonds, after disulfide bonds broke down. This supposition is, however, improbable, because no such increase is registered for reoxydized gluten prepared with alcaline pH at a high carbamide concentration.

The best example for slower reaction of the intramolecular disulfide bonds is presented by the rather intensive test on insuline.

It was shown that of the 3 present disulfide bonds only one could be reduced by thioglycolate. They could disintegrate two disulfide bonds with sodium sulfite but could completely disintegrate only in presence of carbamide, guanidine or phenylmercuric hydroxyde the disulfide bridges. More intensive tests revealed that the intramolecular disulfide bond was the slowest and most difficult of all bonds to react.

Though there is no knowledge available about more complicated proteins and about e.g. the system of the disulfide bonds of gluten proteins by taking into consideration some general regularities and analogies, it may be stated that at least in case of the larger gluten protein molecules, the intramolecular disulfide bonds react slower because of the steric inhibition and are accessible only after a certain loosening up.

Interesting is the observation of BECKWITH and WALL (1966) according to which the amperometrically determined disulfide content of glutenin obtained by sulfite break down, is not more than about two thirds of the actual quantity present up to a carbamide concentration of 3 moles. The total disulfide content can be determined only in a carbamide solution of 6 moles or still higher concentration.

Finally the work of BERNACKA and KACZKOWSKI (1975) must be mentioned, according to which on the basis of reduction dynamics the S-S bonds of the gluten complex may be distributed in four groups (three intermolecular and one intramolecular).

Tests on reduction and reoxydation of gluten indicate the importance of the

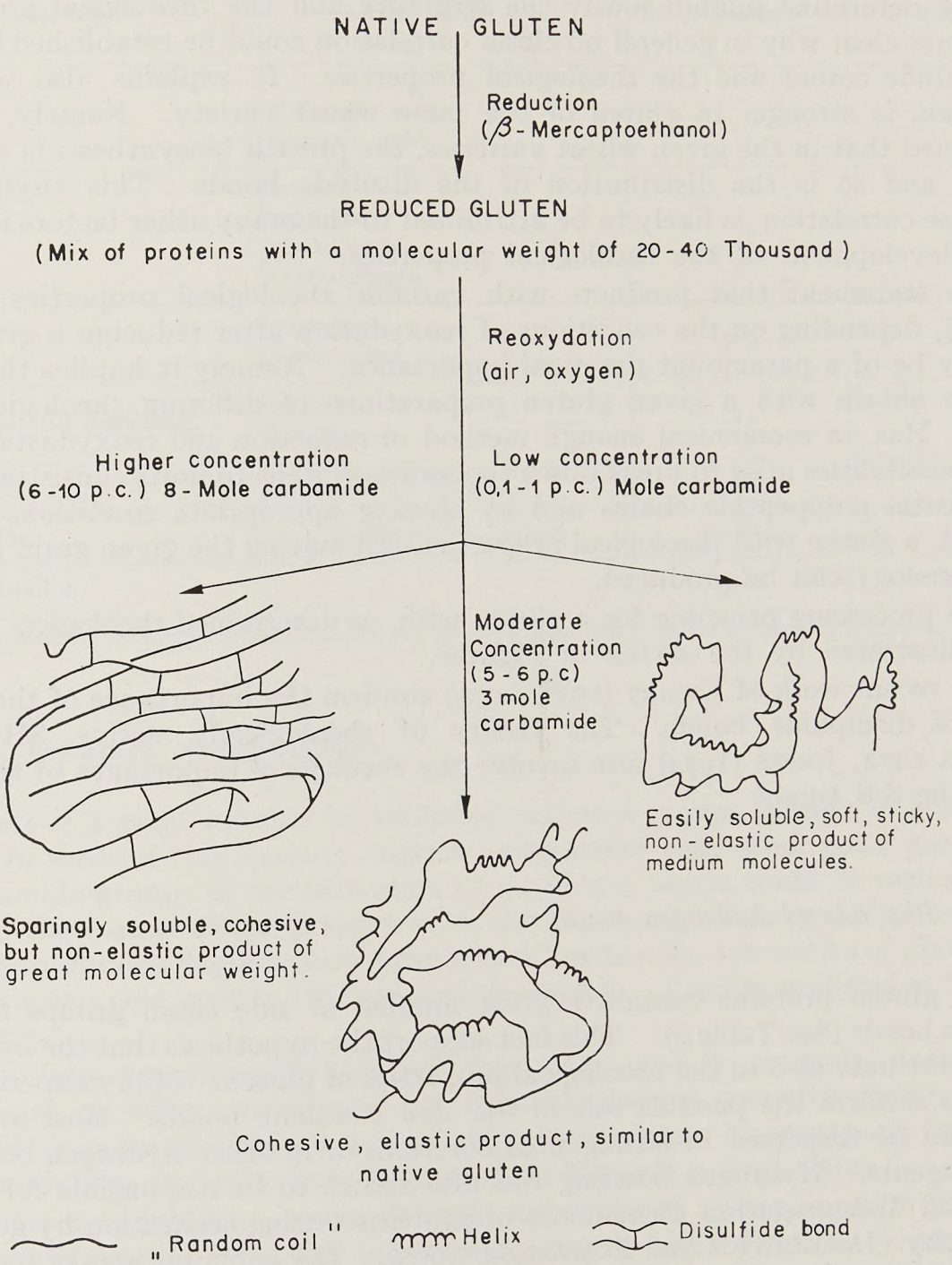


FIG. 8. — *Scheme of the production of gluten with prescribed rheological properties.*

disulfide bonds in the structure of gluten molecules and also in the development of the rheological properties of gluten. Accordingly, the gluten protein fractions of great molecular weights consist of polypeptide chains, connected by disulfide bridges. Beside the disulfide bonds connecting the polypeptide chains, also the number of disulfide bonds within the molecule is of importance. During reduction of the gluten, all the disulfide bonds break down, the obtained product lacks

the rheological properties of the original gluten. When reoxydized, the reversion is practically quantitative. The position of the newly forming bonds, the proportion between inter- and intramolecular disulfide bonds depend on reoxydizing conditions. This fact is also reflected by the rheological properties of the reoxydized product.

It follows from above that the absolute number of the disulfide bonds alone does not determine unambigously the structure and the rheological properties. It becomes clear why in general no closer correlation could be established between the disulfide cotent and the rheological properties. It explains also why the correlation is stronger in gluten of the same wheat variety. Namely, it may be assumed that in the given wheat varieties, the protein biosynthesis is a similar process, and so is the distribution of the disulfide bonds. This anyhow not very close correlation, is likely to be attributed to the many other factors involved in the development of the rheological properties.

The statement that products with variable rheological properties can be obtained, depending on the conditions of reoxydation after reducing a given gluten, may be of a paramount practical importance. Namely it implies the possibility to obtain with a given gluten preparations of differing rheological properties. Has an economical enough method of reduction and reoxydastion been found, possibilities arise to break down the native glutens to units consisting of the fundamental polypeptide chains and by chosing appropriate conditions of reoxydation, a gluten with rheological properties best suiting the given grain processing technology can be produced.

The processing principle for a gluten with so determined rheological properties is illustrated by the sketch in Figure8.

The recent work of EWART (1978, 1979) confirm the importance of the distribution of disulphide bonds. The theory of rheologically active SH-groups BLOKSMA 1972, JONES (1974) also involve the rheological importance of the location of the S-S bonds.

3.32. — *The role of hydrogen bonds*

The gluten proteins contain a great number of side chain groups forming hydrogen bonds (See Table 2). This fact support the hypothesis that the hydrogen bonds contribute also to the rheological properties of gluten. Many experimental facts also confirm the possible role of the non covalent bonds. Most insoluble gluten can be dispersed in strong urea solutions or in other hydrogen bond-disrupting agents. Hydrogen bonding was also shown to be responsible for aggregation and disaggregation phenomena of proteins during separation by gel chromatography (JANKIEWICZ and POMERANZ, 1965). The contributions of hydrogen bonds and reactive sulfurcontaining groups of proteins to the rheological properties of dough were studied by JANKIEWICZ and POMERANZ (1965*a*) using added carbamide and N-ethyl-maleimide. VAKAR *et al.* (1965) reported that freshly washed gluten become stronger and more elastic after dipping in D_2O. These facts indicate also that hydrogen bond plays an important role in the gluten structure. The role of amide groups was studied by HOLME and BRIGGS (1959) and BECKWITH *et al.* (1963). In our laboratory chemically modified glutens were investigated.

TABLE 2

Functional groups in the gluten proteins
(HOLME 1966, POMERANZ 1968)
(*millimoles/100 g protein*)

Group	Amino acids	Gliadin	Glutenin
Acidic	glutamic acid, aspartic acid	27	36
Basic	lysine, arginine, histidine, tryptophane	39	52
Amide	glutamine, asparagine	309	266
Sulfhydryl and disulfide	cysteine, cystine	12	12
Total ionic	(acid + basic)	66	87
Total polar	(hydroxy + amide)	381	365
Total nonpolar		390	301

The influence of deamidation

There is a great number of amidated carboxyl groups present in gluten proteins. In view of this number, high in comparison with other polar groups, the role of amido-groups in the formation of secondary bonds could be rather important. In the course of the present work the penetration indexes of glutens desamidated to various degrees were determined, further the viscosities of gluten solutions, in acetic acid, and in 8 M urea were measured. Results are listed in Tables 3, 4 and 5.

The data unequivocally suggest that, compared to controls, desamidated glutens are of softer consistence, i.e. their rheological properties are less good. The deviations in relative per cent penetrations increase by increasing the degree of desamidation, strongly first, later tending towards a limit value.

As far as solubilities are considered, desamidated gluten is more difficult to dissolve in strongly acid media (below pH 3) but is easily dissolved — in contrast to controls — by phosphate buffer of pH 8. Considering the substantial increase of free carboxyl groups and the acid character of the protein formed this fact seems to be understandable.

On the basis of results of viscosity measurements it can be stated, first, that desamidation primarily affects the viscosities of solutions in acetic acid. According to data in Table 4, this alteration involves the increase of characteristic viscosity, meaning that a change had occurred in the conformation of molecules which produces a more asymmetric structure. To explain this we may suggest that removal of amide groups involves elimination of secondary, e.g. hydrogen

TABLE 3

Rheological properties of desamidated gluten

Sample No.	Difference in penetration in reference to controls Δ P (p. 100)					
	degree of desamidation (p. 100)					
	10	30	50	70	90	100
1 . . .	15	24	31	39	39	42
2 . . .	12	23	35	46	47	50
3 . . .	17	29	39	51	55	57
4 . . .	10	21	31	35	34	39
5 . . .	8	17	29	37	42	42
6 . . .	7	23	29	35	37	39
7 . . .	13	21	34	45	49	53
8 . . .	15	21	29	42	47	48
9 . . .	11	19	33	42	47	48
10 . . .	14	23	32	44	43	43

TABLE 4

Characteristic viscosity of desamidated gluten solutions in acetic acid medium

Ser. No.	(η) dl/g						Acetic acid concentration
	degree of desamidation (p. 100)						
	10	30	50	70	90	100	
1 . . .	0.425	0.445	0.462	0.480	0.505	0.510	0.05 N
	0.430	0.435	0.440	0.437	0.435	0.420	1.0 N
2 . . .	0.480	0.485	0.492	0.507	0.580	0.510	0.05 N
	0.475	0.480	0.481	0.475	0.470	0.469	1.0 N
3 . . .	0.460	0.475	0.490	0.502	0.500	0.499	0.05 N
	0.462	0.467	0.470	0.465	0.459	0.458	1.0 N
4 . . .	0.502	0.520	0.530	0.536	0.535	0.537	0.05 N
	0.490	0.501	0.506	0.508	0.506	0.501	1.0 N
5 . . .	0.397	0.412	0.431	0.432	0.430	0.429	0.05 N
	0.400	0.405	0.405	0.420	0.400	0.390	1.0 N

TABLE 5

Characteristic viscosity of the solutions of desamidated gluten in 8 M urea

Ser. No.	(η) dl/g					
	degree of desamidation (p. 100)					
	10	30	50	70	90	100
1 . . .	0.480	0.475	0.475	0.478	0.468	0.450
2 . . .	0.550	0.552	0.545	0.547	0.530	0.527
3 . . .	0.501	0.497	0.502	0.504	0.486	0.479
4 . . .	0.560	0.550	0.552	0.540	0.531	0.535
5 . . .	0.480	0.482	0.480	0.470	0.447	0.450

bonds in consequence of which a more loose structure will emerge. Presumably, due to the dissociation of carboxyl groups set free, within a molecule, between groups with identical charge, repulsive forces are operative. This supposition is supported by the experimental finding that increase of viscosity is substantially less in 1.0 N acetic acid at higher pH and lower dissociation, or that on these conditions sometimes no increase of viscosity is found at all. Probably on the one hand electrostatic repulsion is weaker, on the other hand new hydrogen bonds are formed between spatially favourably situated groups.

Similar conclusions can be drawm from viscosity data relating to glutens, and to desamidated glutens dissolved in 8 M urea. A comparison of viscosity data of solutions in 0.05 N acetic acid and in 8 M urea reveals that in reference to the acetic acid solution increase of viscosity of not desamidated controls is significantly higher than that of partially desamidated samples. The deviation can be explained on the basis that in amidated glutens there are substantially more hydrogen bonds present, and these are disrupted by the urea added and thus the conformations are altered.

At lower pH (1 N acetic acid) also desamidated gluten samples show viscosity data widely divergent from those of solutions with urea, sometimes these values are very nearly the same as those for the controls; and this finding supports the idea of new hydrogen bonds being formed, as mentioned in the discussion of data shown in Table 4.

Effect of esterification

Esterification is one of the possibilities to transform the free carboxyl groups. The partial conversion of amide groups into esters is also feasible. In the course of our experiments the rheological properties of glutens esterified with methanol or ethanol, have been studied, together with the viscosity of the solutions prepared from the derivatives with 0.05 N acetic acid. Results are listed in Tables 6 and 7.

Data from Table 6 as show that by increasing the degree of esterification the rheological properties of gluten change for the worse relaxation time will be signi-

TABLE 6

Relaxation of gluten esterified with methanol

Ser. No.	Relaxation time, sec.						
	extent of methylation, mmole/g						
	0	0.30	0.50	1.0	1.5	2.0	3.00
1	82	79	60	54	48	45	46
2	45	44	36	30	28	25	26
3	73	70	60	52	45	40	41
4	55	56	51	41	35	32	34
5	69	65	54	48	40	36	30

TABLE 7

Viscosity of solutions of gluten esterified with methanol

Ser. No.	(η) dl/g						
	level of methylation mmole/g						
	0	0.3	0.5	1.0	1.5	2.0	3.0
1	0.442	0.439	0.384	0.350	0.321	0.295	0.280
2	0.495	0.480	0.401	0.362	0.318	0.288	0.275
3	0.480	0.469	0.360	0.331	0.297	0.291	0.302
4	0.530	0.529	0.460	0.431	0.390	0.321	0.305
5	0.420	0.415	0.340	0.291	0.270	0.258	0.261

ficantly shorter. During the first period, which corresponds to the esterification of the free carboxyl present, no essential change occurs; this suggests that the role of free carboxyls in the formation of secondary bonds is not significant.

Viscosity data unequivocally show that characteristic viscosity is very much reduced, this argues for more compact and less asymmetric molecules. The alteration consists perhaps therein that alkylated protein is highly hydrophobe, thus, in water, the degree of its hydratation will be lower.

Effect of acylation

In order to study the rheological properties of acylated gluten, the penetration figures of the hydrated gluten derivative were established, and the viscosities of their solutions in 0.1 N acetic acid were measured. Results are summarized in Tables 8 and 9.

Data reveal that the rheological properties of acylated gluten are very much inferior to those of native gluten. The decrease of cohesivity suggests that primary amino groups play a substantial role in the formation of intermolecular noncovalent bonds.

TABLE 8

Penetration figures of acylated gluten

Ser. No.	Penetration, 0.1 mm			
	per cent of acylation			
	0	40	80	100
1	72	142	152	160
2	58	108	112	135
3	85	120	135	142
4	70	105	121	143
5	94	143	162	180
6	45	92	108	125
7	48	89	102	117
8	73	104	130	135

TABLE 9

Characteristic viscosity of the solutions of acylated gluten

Ser. No.	(η) dl/g			
	per cent of acylation			
	0	40	80	100
1	0.425	0.396	0.392	0.388
2	0.480	0.428	0.420	0.422
3	0.460	0.432	0.417	0.418
4	0.502	0.477	0.472	0.465
5	0.397	0.368	0.362	0.365
6	0.485	0.444	0.427	0.430
7	0.510	0.482	0.469	0.461
8	0.447	0.417	0.405	0.396

Experimental results show that no major alteration in viscosity takes place; after an initial small decrease the viscosity values remain practically constant. Thus it can be concluded that no important change of molecular conformation occurs, or that primary amino groups participate principally in the formation of intermolecular bonds.

3.33. — *Effect of hydrophobic bonds*

Gluten proteins contain several amino acids which possess hydrophobic side chains (alanine, leucine, phanylalanine, isoleucine, valine, proline). Moreover, taking into consideration that the hydrophobic parts of longer polar side chains (e.g. in the case of lysine and glutamic acid) may also interact, there can be no

doubt about the potential possibility of the formation of hydrophobic bonds.

Dough and gluten formation proceed in aqueous media. Owing to the fact that an interaction of the non-polar groups with water is " unfavourable " from the thermodynamic viewpoint, the thermodynamic tendency points towards a linkage of the non-polar groups with each other (with a consequent weakening of the interaction between these groups and water). This problem is dealt with in the review by KAUZMAN (1959). In general, the formation of hydrophobic bonds is an endothermic process, i.e. the change in thermodynamic potential is negative since the effect of the change in entropy ($T\Delta S$) exceeds that of the change in enthalpy (ΔH). Up to a certain temperature limit, the strength of hydrophobic bonds increases with increasing temperature, so that hydrophobic bonds are of particular importance from the viewpoint of thermal stability of proteins.

The solubility of gliadin in non-polar solvents, and the influence of the latter on solubility are also indicative of the possible importance of the role of hydrophobic bonds. All this shows convincingly that a study of the hydrophobic bonds is unavoidably necessary for the understanding of factors which influence the structure and rheological properties of gluten proteins.

Up to now wheat protein research paid little attention to this problem. Only the observation might perhaps be mentioned that the rheological properties of doughs are changed already by small quantities of certain aliphatic hydrocarbons (MUELLER *et al.* (1965), PONTE *et al.* (1964, 1966, 1967)).

Indirect methods have been used primarily in the present work. The effect of compounds has been studied, which are able to interact with the hydrophobic groups of the gluten complex, and through these groups can interact with the existing hydrophobic bonds.

Effect of hydrocarbons on the rheological properties of gluten

These tests were carried out as follows. Dehydrated gluten was contacted with water containing a known amount of hydrocarbons. After hydratation and swelling, the hydrated mass was worked mechanically, until a continuous, homogeneous substance was obtained. The excess solution was removed, and the relaxation test was performed as described earlier. The experimental results are shown in Table 10.

The data in Table 10 show that the rheological properties of gluten are affected unfavourably by the presence of higher aliphatic hydrocarbons. When tested organoleptically, gluten becomes less stretchable and crumbly. In the case of pentane and hexane, an increase in the relaxation time add in the force necessary to cause a deformation of identical degree can be observed, particularly with glutens of poorer quality.

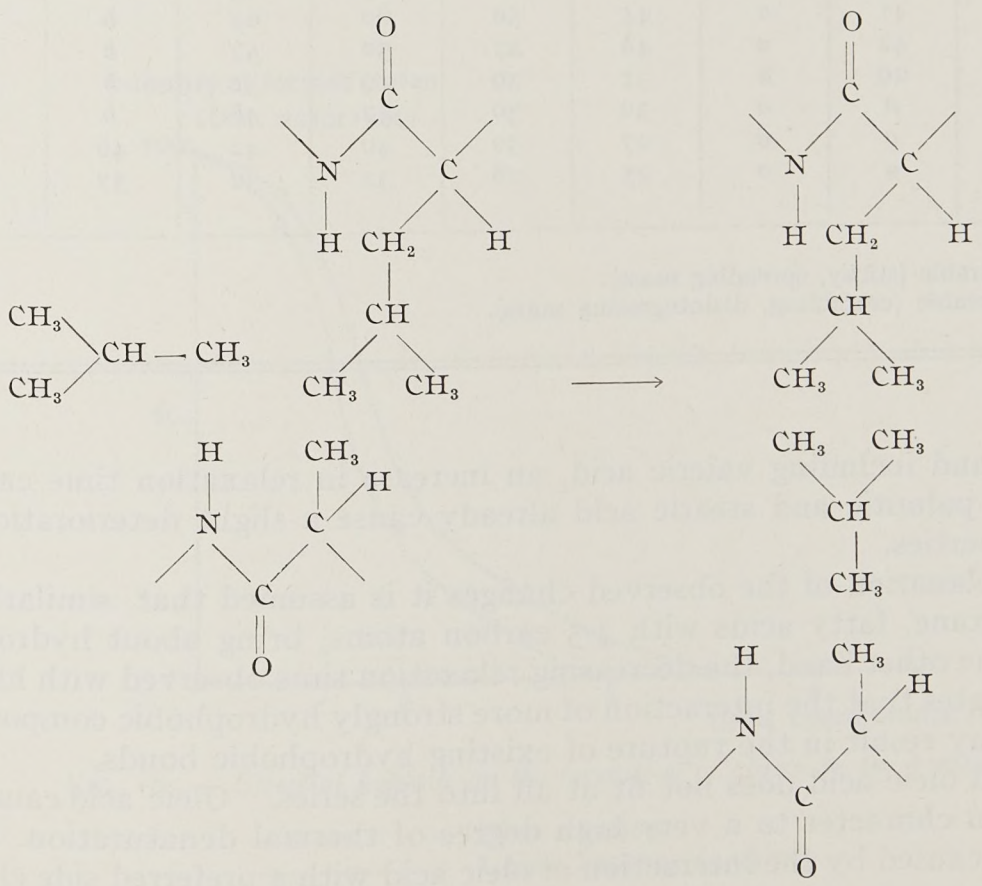
The pronounced changes which can be detected even at the relatively low concentrations used, definitely indicate an interaction between the hydrocarbons and the proteins of gluten. As concerns the character of this interaction, on the basis of thermodynamical considerations one can assume that a linkage is formed between the hydrocarbons and the hydrophobic side chains of proteins. In the case of pentane and hexane, a weaker bond is formed, extending or rather protecting those hydrophobic nuclei which in the course of hydratation, osmotic uptake and swelling, and peptization prevent the aggregates from unlimited swelling and disintegration. When higher hydrocarbons are added, the interaction may become stronger due to the higher affinity, so that existing interactions between side chains

TABLE 10

Effect of hydrocarbons on the rheological properties of gluten

No.	Relaxation time (sec.)					
	Control	Pentane	Hexane	Heptane	Octane	Undecane
	0.03 mole/100 g of gluten					
1 . . .	102	106	101	94	82	65
2 . . .	95	95	96	82	76	72
3 . . .	88	91	82	71	66	50
4 . . .	73	72	70	72	68	53
5 . . .	69	65	63	58	55	48
6 . . .	62	65	60	55	54	45
7 . . .	58	60	62	59	51	45
8 . . .	53	48	49	44	40	37
9 . . .	43	44	40	38	37	35
10 . . .	38	45	40	38	40	41

may cease, i.e. existing hydrophobic bonds may be ruptured, and replaced by bonds between the added hydrocarbon and the side chains. This situation is analogous to that assumed for the rupture of the hydrogen bonds by urea. The two analogous processes are illustrated by the following scheme:



Effect of fatty acids on the rheological properties of gluten

The procedure was the same as in the preceding series of experiments with the difference that part of the fatty acids was present in the aqueous medium as an emulsion.

The rheological properties of the glutens under these conditions are summarized in Table II.

The data in the table show an interesting pattern. The changes in rheological properties are different depending on the acid and the gluten. In the case of formic and acetic acid, the peptizing effect predominates, which brings about rapid deterioration of the rheological properties. With acids of increasing carbon atom

TABLE II

Effect of fatty acids on the rheological properties of gluten

No.	Relaxation time (sec.)								
	Acid added: 0.01 molar solution (emulsion)								
	Control	Formic	Acetic	Propionic	Butyric	Valeric	Palmitic	Oleic	Stearic
1 . . .	102	39	40	56	72	85	80	*b*	75
2 . . .	95	47	30	50	68	80	82	*b*	71
3 . . .	88	39	28	52	59	69	79	*b*	80
4 . . .	73	48	*a*	41	48	53	63	*b*	65
5 . . .	69	41	*a*	44	50	60	62	*b*	58
6 . . .	62	42	*a*	48	47	50	52	*b*	47
7 . . .	58	29	*a*	32	39	40	52	*b*	49
8 . . .	52	*a*	*a*	30	30	42	48	*b*	41
9 . . .	43	*a*	*a*	27	30	40	42	46	30
10 . . .	38	*a*	*a*	25	28	32	32	57	28

a = not measurable (sticky, spreading mass).
b = not measurable (crumbling, disintegrating mass).

number, up to and including valeric acid, an increase in relaxation time can be observed, while palmitic and stearic acid already cause a slight deterioration of rheological properties.

For the explanation of the observed changes it is assumed that, similarly to pentane and hexane, fatty acids with 3-5 carbon atoms, bring about hydrophobization. On the other hand, the decreasing relaxation time observed with higher fatty acids indicates that the interaction of more strongly hydrophobic compounds with proteins may result in the rupture of existing hydrophobic bonds.

Unsaturated oleic acid does not fit at all into the series. Oleic acid causes a change similar in character to a very high degree of thermal denaturation. Presumably, this is caused by the interaction of oleic acid with a preferred side chain.

Effect of hydrocarbons on the formation of gluten in the presence of urea

In earlier works concerned with the role of hydrogen bonds, the effect of the addition of increasing amounts of urea on the rehydration of dry gluten and on gluten formation has been studied. These experiments were now repeated with the difference that various hydrocarbons were added to the urea solution (0.03 mole

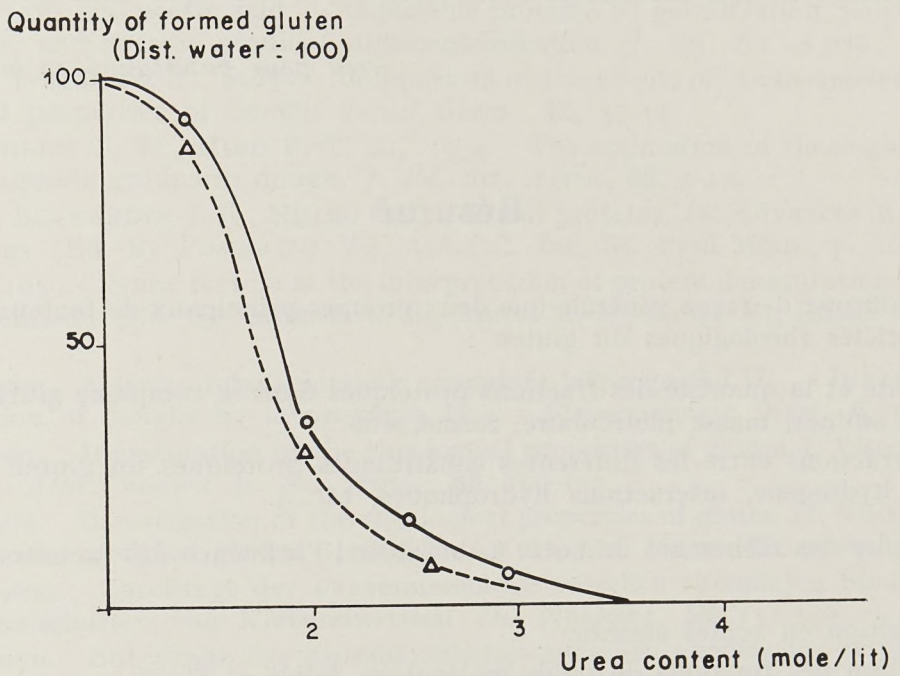


FIG. 9. — *Effect of octane on the formation of gluten in the presence of urea.*

– – – △ – – – Urea solution.
——— o ——— Urea+octane (0,03 m).

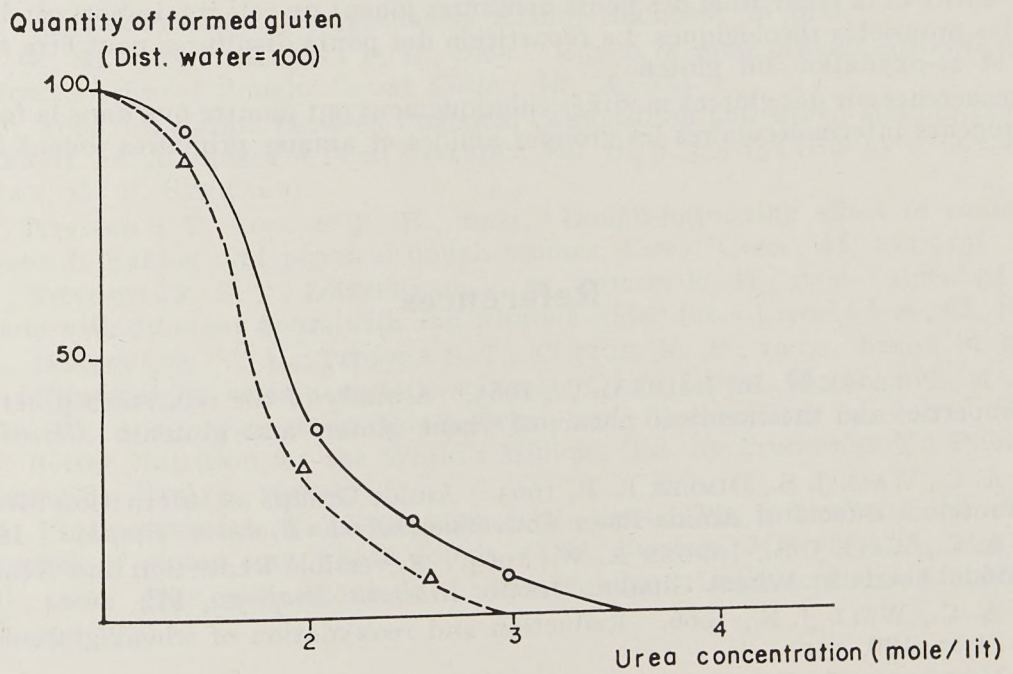


FIG. 10. — *Effect of heptane on the formation of gluten in the presence of urea.*

– – – △ – – – Urea solution.
——— o ——— Urea+heptane (0,03 m).

per 1 g of gluten). The experimental results show that under these conditions, the absence of gluten formation or a considerable decrease in the amount of gluten formed is observed already at a lower urea concentration. The results are plotted in Figure 9 and 10.

The observed behaviour can be explained by assuming that the combined addition of urea and hydrocarbons hinders not only the formation of intermolecular hydrogen bonds but also the formation of the corresponding hydrophobic bonds as a result of the interaction between hydrocarbons and nonpolar protein side chains.

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Résumé

On peut affirmer de façon générale que deux groupes principaux de facteurs peuvent influencer les propriétés rhéologiques du gluten :

— la qualité et la quantité des fractions protéiques dans le complexe glutineux (composition en acides aminés, masse moléculaire, forme, etc...);

— les interactions entre les différentes constituants protéiques du gluten (liaisons disulfures, liaisons hydrogène, interactions hydrophobes, etc...).

Dans le cadre des recherches de notre Laboratoire, l'influence des facteurs suivants a été examinée :

— composition en acides aminés;

— proportion des fractions de poids moléculaire faible et élevé;

— interaction entre des constituants protéiques.

Il a été démontré que la teneur en cystine, le degré d'amidation et le contenu en résidus hydrophobes d'acides aminés ont une incidence significative sur les propriétés rhéologiques.

Les fractions de poids moléculaire faible, estimées à l'aide de la chromatographie sur gel, électrophorèse en gel, ou peptisation, diminuent les qualités rhéologiques du gluten.

La quantité et la répartition des ponts disulfures jouent un rôle très important dans la détermination des propriétés rhéologiques. La répartition des ponts disulfures peut être modifiée par réduction et re-oxydation du gluten.

Des recherches sur des glutens modifiés chimiquement ont montré que dans la formation des ponts hydrogènes intermoléculaires les groupes amides et amines primaires jouent le rôle principal.

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Chemical modification as a probe of gluten structure

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Summary

The reactivity of a protein-bound amino acid toward chemical modification is a measure of the accessibility of that residue. This gives an indication of its location and role in the folded structure of the protein molecule. A variety of chemical reagents has been employed to modify amino acid residues in wheat gluten. The basic amino acids, lysine and arginine, are extensively modified by potassium cyanate and 1,2-cyclohexanedione respectively. Some cystine residues, particularly those in the gliadin fraction, react very quickly with sodium hydroxide, but the cystines in glutenin are much less reactive. Tyrosine residues react slowly with N-acetylimidazole under non denaturing conditions, but more rapidly in strong urea solutions. These results suggest that the conformation of gluten proteins is typical of proteins in general with the charged residues on the surface of the molecule and the hydrophobic residues in the interior. The cystine residues of gliadin are apparently more accessible than those of glutenin. The molecular weight ranges of gluten, and alkali-treated glutens in which the disulphide bridges are broken, suggest that disulphides do not play the dominant role in the factors which determine the size of aggregates of gluten subunits.

Key words : *gluten — amino acids — molecular conformation.*

The sequence and three dimensional location of amino acid residues play an important role in determining the physical and chemical properties of proteins. Residues which are located in the interior of the protein interact to a lesser extent with the solvent, with other protein molecules and with dissolved solutes than residues on the surface. The susceptibility to chemical modification of different amino acid residues in a protein is therefore a means of determining the relative locations of specific residues. Surface groups would be expected to have reactivities similar to that of the same groups in a simple peptide, while buried groups would have lower reactivities. There are exceptions to this pattern, brought about by a variety of factors, but in general the information obtained by this method correlates well with other methods such as X-ray analysis.

Where the primary sequence of a particular protein is known, it is possible to consider the reactivity of each amino acid residue individually. On the other hand, if the sequence of a protein is not known, or if the protein consists of a

heterogeneous mixture of individual protein molecules, the information gained about each type of amina acid is necessarily more generalized.

Hydrophobic residues have been shown to have a tendency to occupy the interior of proteins, where their interactions with aqueous solvents are minimized. Hydrophilic residues which are charged around neutral pH — lysine, arginine, glutamic and aspartic acids, and to a lesser extent histidine — normally occupy surface positions where they are readily solvated by water, thus aiding the solvation of the entire protein. Between these two extremes lie those amino acids which, although uncharged and relatively non-polar, are functionalized with hydroxyl, amide, phenolic and sulphydryl groups. These, by the virtue of their hydrogen bonding capability may usefully contribute to the surface or interior structure of a protein.

Unfortunately, selective reagents do not exist for each of the commonly occurring amino acids. Amino acids with non-functionalized aliphatic side chains, such as leucine, *iso*-leucine, valine and alanine, are unreactive towards most reagents. The exceptions are those which are quite non-specific such as free radicals. Nevertheless, the modification of proteins with specific chemical reagents can provide useful information about a protein's structure.

Because of its heterogeneous nature, very little work has been done on the chemical modification of gluten. Previous work has been directed towards altering its functional properties for potential industrial purposes (Simmonds *et al.*, 1973) and no attention has been given to modifications which retain its native state intact as far as possible. The study to be described here has been designed to investigate the reactivity of certain amino acids towards chemical reagents in the protein's natural conformation. The results have given some indication of the organization of the amino acids within the protein complex comprising gluten.

Experimental section

Materials

Gluten used in the experiments was obtained from two sources. Commercial gluten was a gift of Manildra Starches Pty Ltd, Auburn, N.S.W. Handwashed gluten was prepared from flour milled from the hard wheat variety Songlen. 1,2-Cyclohexanedione was prepared by selenium dioxide oxidation of cyclohexanone (Hach *et al.*, 1963). Potassium cyanate (BDH) and sodium hydroxide (Ajax Univar) were purchased from Anax, North Ryde, N.S.W. N-Acetylimidazole was prepared by direct acetylation of imidazole with acetic anhydride (Reddy *et al.*, 1963).

Methods

Gluten from each source was subjected to reaction conditions as described below. The results of modification of each sample were the same within experimental limits, unless otherwise stated.

Reaction of gluten with sodium hydroxide

Gluten (20 g) was added with stirring to 0.2 M aqueous sodium hydroxide (125 ml). Stirring was continued until after neutralization was effected as rapidly as possible by the addition of 2 M hydrochloric acid. The mixture was dialysed against distilled water at 4 °C and lyophilized.

Reaction of gluten with 1,2-cyclohexanedione

(*a*) Gluten (25 g) was dispersed in 0.01 M aqueous sodium hydroxide, and 1,2-cyclohexanedione (4 g) was added. The mixture was allowed to stand for 16 hours. The initial pH of the reaction mixture was 11.0, and dropped to a final pH of 10.7. The mixture was neutralized with hydrochloric acid, dialysed against distilled water at 4 °C and lyophilized.

(*b*) Gluten (40 g) was dispersed in 0.2 M sodium borate buffer, pH 9.0, and 1,2-cyclohexanedione was added. The mixture was stirred for three hours before acetic acid (10 ml) was added to terminate the reaction. The reaction mixture was dialysed against distilled water at 4 °C and lyophilized.

Reaction of gluten with potassium cyanate

Gluten (20 g) was suspended in 0.05 M sodium borate buffer, pH 8 (500 ml). Potassium cyanate (40.56 g) was added to give a final concentration of 0.5 M. The stirring was continued for two hours while the pH was maintained in the range 8.0 to 8.1. The mixture was dialysed overnight at 4 °C and lyophilized.

Reaction of gluten with N-acetylimidazole

Gluten (0.8 g) was suspended in 0.05 M sodium borate, pH 8 (100 ml), and incubated with N-acetylimidazole (600 mg) at 25 °C for one hour. The mixture was adjusted to pH 4.5 with acetic acid, dialysed against distilled water and lyophilized.

Control experiments

In each of the reactions with 1,2-cyclohexanedione, potassium cyanate or N-acetylimidazole described above, a gluten sample was subjected to the reaction conditions in the absence of the chemical reagent. The results of the modification experiments were compared with those of the appropriate control.

Results

Effect of sodium hydroxide on gluten

When gluten is treated with sodium hydroxide solution, a number of chemical reactions take place depending upon the conditions of reaction. In 0.2 M alkali, the protein is denatured almost instantaneously at room temperature, as mea-

sured by its mixing and baking properties (Fig. 1). The effect on its chemical properties, however, does not take place as rapidly. The amino acid composition of gluten which has been dispersed in 0.2 M sodium hydroxide at 25° and then neutralized as quickly as possible is not affected except for a small reduction in the amount of cystine present. This reduction is equivalent to approximately one fifth (1/5) of a disulphide group for a protein of molecular weight 100 000. Although small, this loss can be measured reproducibly, within experimental limitations. After treatment with 0.2 M sodium hydroxide for ten minutes, the reduction in cystine content is equivalent to one disulphide group for a protein of molecular weight 100 000. Longer times and higher temperatures of reaction cause greater loss of cystine (Table 1).

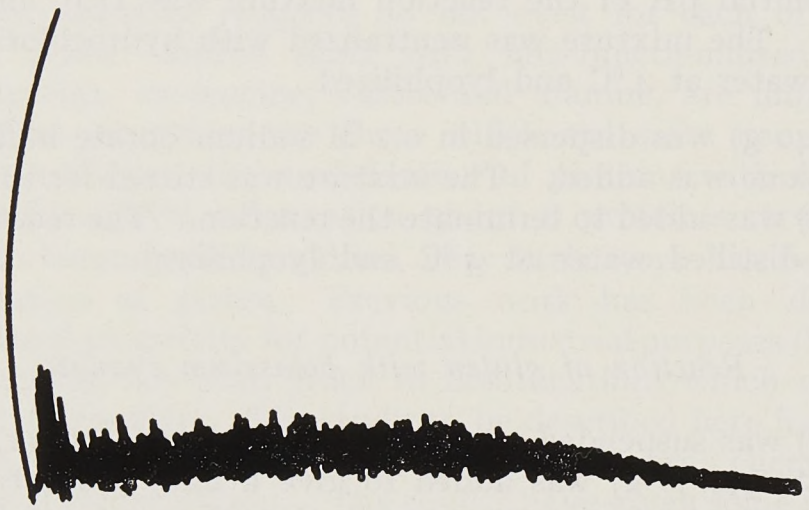

FIG. 1. — *Mixogram of a reconstituted flour containing 29.75 g starch and 5.25 g alkali-treated gluten (0 min. time sample).*

TABLE I

Effect of sodium hydroxide on the cystine and the primary amide contents of gluten proteins

Reaction conditions	Amount of cystine[b] (p. 100)	Amount of deamidation[a] (p. 100)
25 °C 0 min.[c]	97	0
10 min.	92	0
20 min.	88	0
1 hour	na	1
6 hours	trace	4
40 °C 0 min.[c]	92	0
1 hour	66	2
4 hours	trace	na
6 hours	0	6

a Measured as number of micromoles cystine remaining as a percentage of the initial cystine content.

b Measured as the number of micromoles of ammonia lost as a percentage of the initial ammonia content.

c 0 min. reaction refers to a reaction mixture in which gluten was dispersed and then was neutralized as rapidly as possible.

Other chemical properties are not altered as rapidly. Deamidation of the large numbers of glutamine and asparagine residues occurs slowly. After a reaction time of two hours at room temperature, approximately one percent of the primary amides is eliminated (Table 1). At 40 °C, deamidation is more rapid, but after six hours there is still a reduction of only six p. 100 of the amide groups.

MacRitchie (1978) has shown that gluten can be separated into a gliadin and a glutenin fraction by extraction with dilute acids. By similarly extracting alkali-treated gluten, it has been found possible to separate gliadin and glutenin from the denatured protein. The proportions of each component thus recovered are independent of whether the gluten has been alkali treated or not. Except

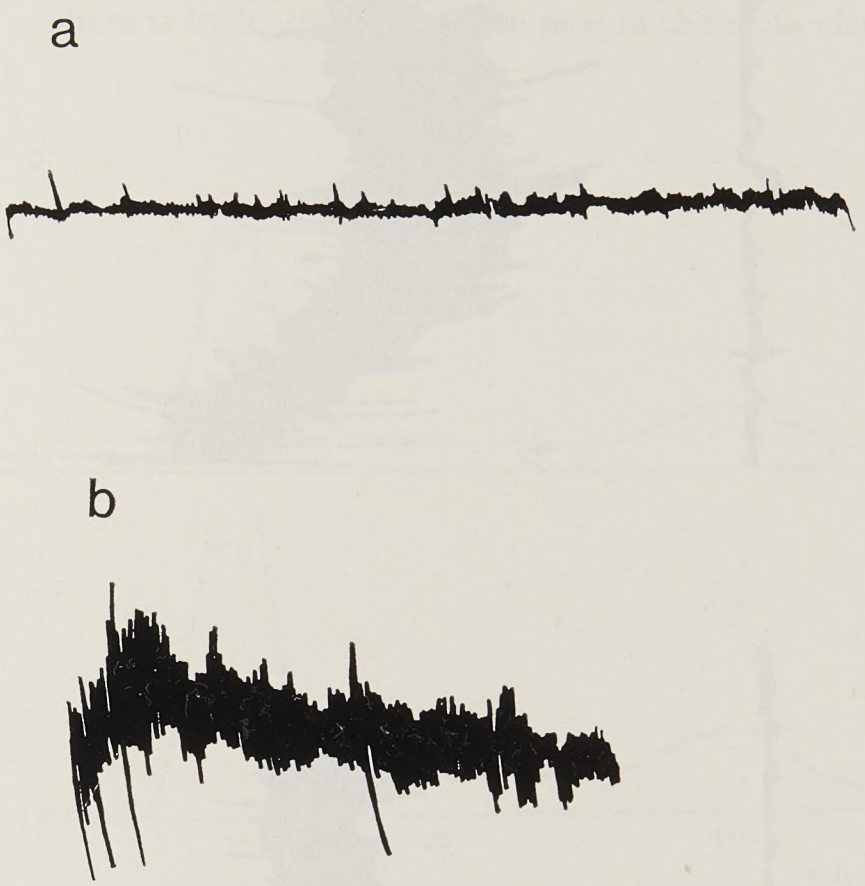

Fig. 2. — *Mixograms of reconstituted flours containing 29.75 g starch and (a) 3.00 g alkali-treated gliadin (10 min. reaction) and 2.25 g unreacted glutenin; (b) 3.00 g unreacted gliadin and 2.25 g alkali-treated glutenin (4 hr. reaction).*

for the changes already described for whole gluten, the amino acid composition for each component is unchanged. This observation indicates that it is the same fractions that are being isolated both before and after alkali treatment.

The two components from untreated gluten have been reacted separately with sodium hydroxide, and the effect of alkali on these proteins is illustrated in Figure 2. Apparently it is the gliadin component whose properties are destroyed rapidly, while the glutenin is only attacked over a longer period of time.

When alkali-treated gluten is fractionated by the method of MacRitchie (1978), the gliadin fraction which is isolated has lost its mixing and baking properties. The mixograms of reconstituted flours using alkali-treated gluten, and the gliadin and glutenin components isolated from it are shown in Figure 3. The mixogram of alkali-treated gluten (Fig. 3*a*) is equivalent to that of gliadin from alkali-treated gluten plus normal glutenin (Fig. 3*b*), while the mixogram

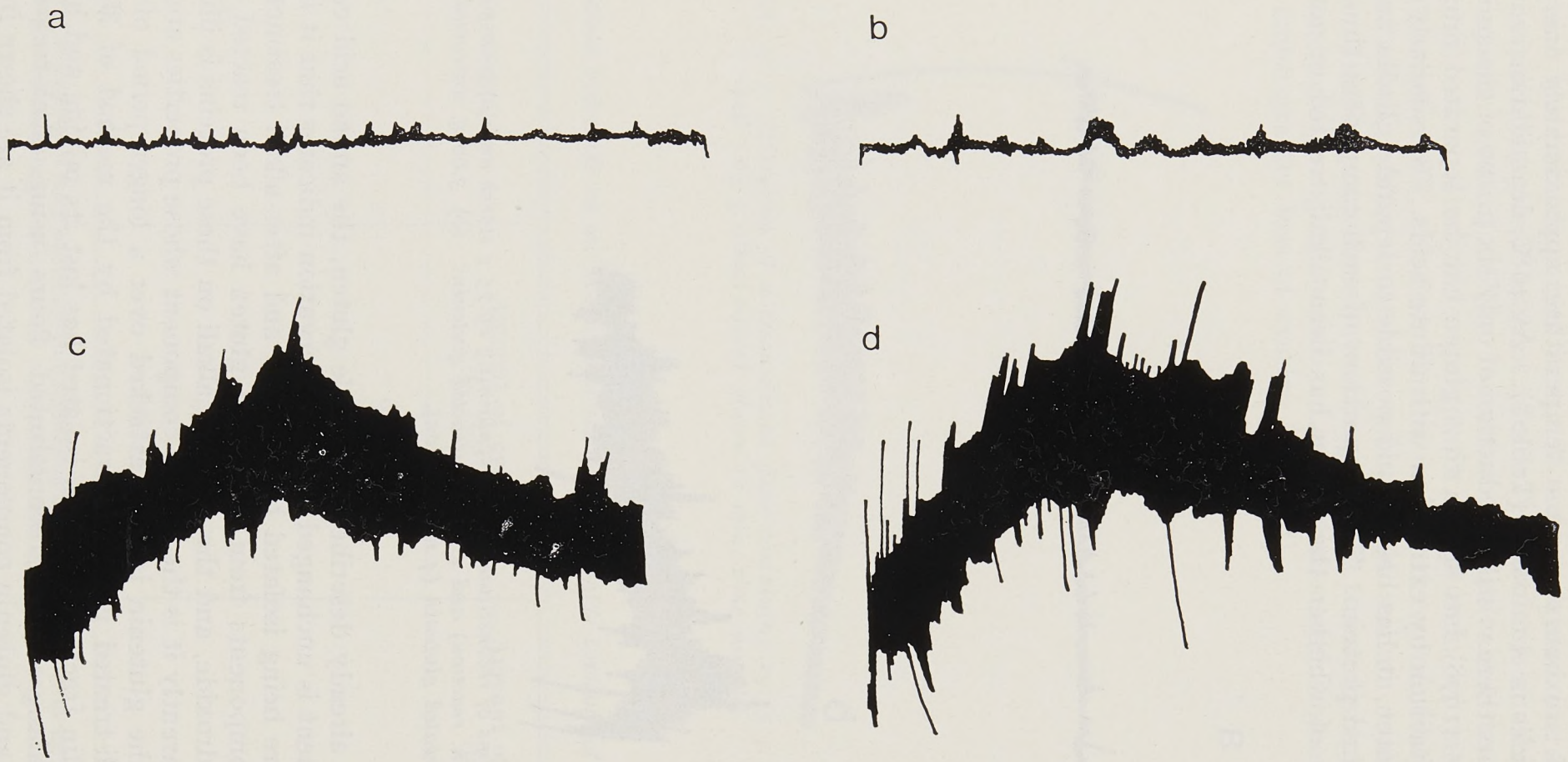

FIG. 3. — *Mixograms of reconstituted flours containing 29.75 g starch and (a) 5.25 g alkali-treated gluten (10 min. reaction); (b) 3.00 g gliadin extracted from alkali-treated gluten (10 min. reaction) and 2.25 g glutenin extracted from unreacted gluten; (c) 3.00 g gliadin from unreacted gluten and 2.25 g glutenine from alkali-treated gluten (10 min. reaction); (d) 5.25 g unreacted gluten.*

using normal gliadin plus glutenin from alkali-treated gluten (Fig. 3*c*) is essentially unchanged from that of normal gliadin plus normal glutenin (Fig. 3*d*). This result demonstrates clearly that in whole gluten it is the gliadin fraction which is particularly susceptible to alkali treatment.

Molecular weight range of alkali-treated gluten

The elution patterns of gluten derivatives subjected to gel filtration on Sephadex G-150 are shown in Figure 4. The sample of alkali-treated gluten was one which had been treated for six hours at 25 °C, and contained no residual disulphide groups. There is little difference to be seen in the molecular weight range

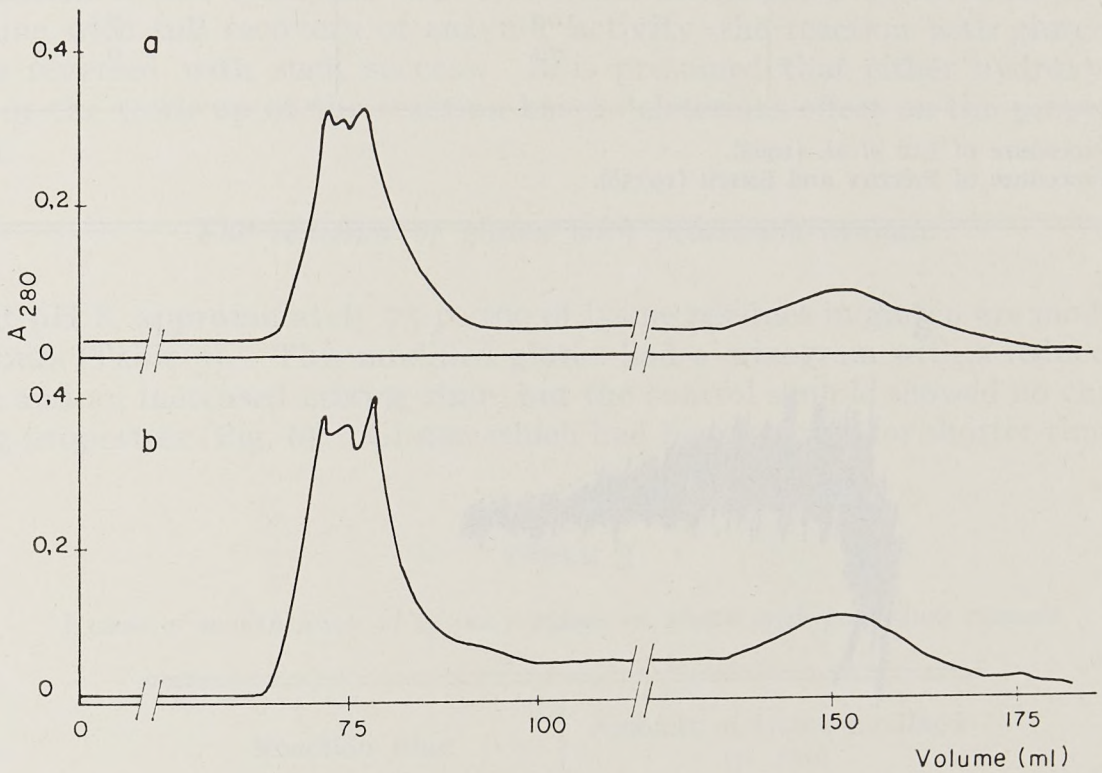

FIG. 4. — *Elution pattern of gel filtration on Sephadex G-150 in 3 M urea — 0.1 M acetic acid — 0.01 M cetyltrimethyl ammonium bromide of (a) unreacted vital gluten; (b) alkali-treated gluten (six hours at 40 °C).*

of the alkali-treated gluten and of the untreated gluten, and the small differences that are observed are probably a result of the *exo*-cleavage of short oligo-peptides from the ends of the protein molecules. Likewise, the molecular weight ranges of alkali-treated gliadin and alkali-treated glutenin appear to be very similar to their unreacted counterparts.

The reaction of gluten with 1,2-cyclohexanedione

Arginine residues in gluten react extensively with 1,2-cyclohexanedione using both procedures described in the experimental section. Table 2 shows the amount of arginine and lysine remaining after reaction according to each of the procedures has been carried out. After 16 hours at pH 11, an intense yellow-green

coloration developed, indicating that, as well as the modification of arginine residues, some modification of lysine residues had occurred. After acid hydrolysis of the modified protein, amino acid analysis showed only a small reduction in the amount of lysine present compared with an almost total loss of arginine. The observed loss of lysine may have been small for two reasons. The product derived

TABLE 2

Extent of modification of arginine and lysine residues in gluten with 1,2-cyclohexanedione

Reaction conditions	Amount of arginine modified (p. 100)	Amount of lysine modified (p. 100)
pH 11[a]	100	7
pH 9b[b]	72	0

a Procedure of Liu *et al.* (1968).
b Procedure of Patthy and Smith (1975*b*).

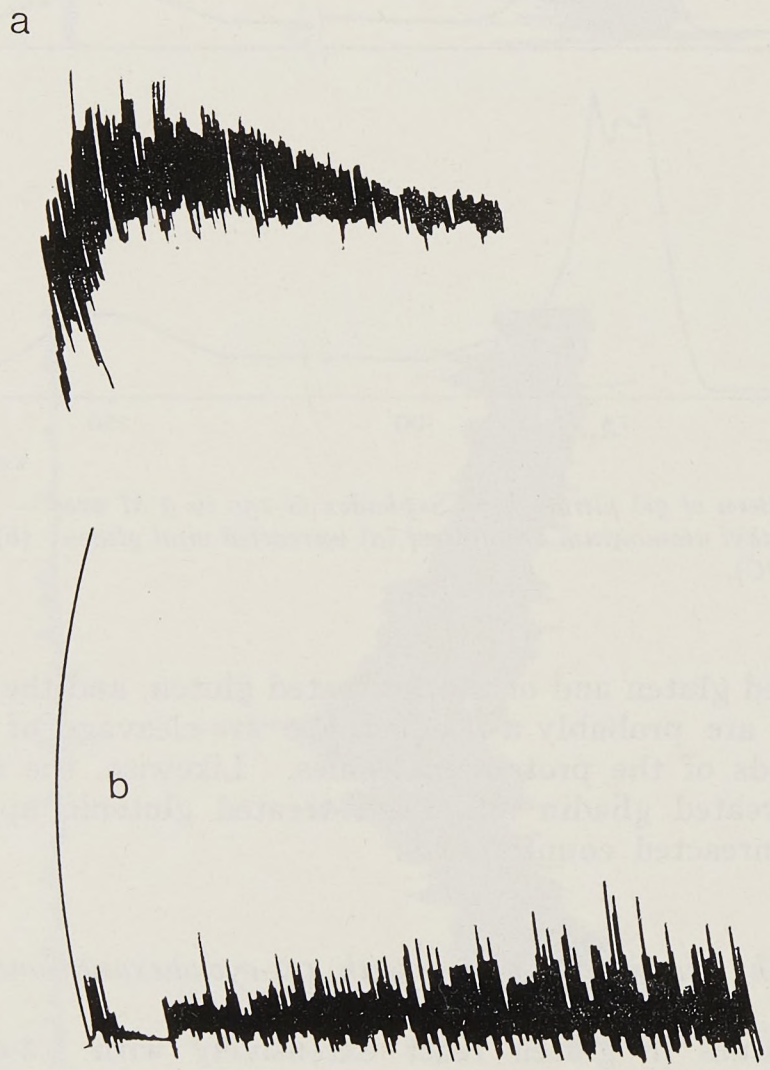

Fig. 5. — *Mixograms of reconstituted flours containing 29.75 g starch and (a) 5.5 g "control" gluten for cyclohexanedione modification, pH 9; (b) 5.25 g cyclohexanedione modified gluten, pH 9.*

from modification of lysine may revert to the unmodified amino acid during acid hydrolysis, or the colour arising from the product may be very intense and misleading with regard to the actual number of lysine residues modified.

Because of its specificity for arginine residues, the method of PATTHY and SMITH (1975*a*) circumvents the problem of concurrent lysine modification. In addition, by conducting the reaction in borate buffer, the modified arginine product may be converted back to arginine using hydroxylamine or hydrazine. When gluten is treated according to this method, the reaction is not as extensive as in the first case (Table 2). However a dramatic effect is observed on the mixogram of a reconstituted flour containing the arginine-modified protein (Fig. 5). The mixogram of the control gluten has deteriorated from that of unreacted gluten, but the mixogram of the modified gluten looks completely unrelated.

Although PATTHY and SMITH (1975*b*) observed that the reaction of both ribonuclease A and lysozyme with cyclohexanedione could be reversed by hydroxylamine with full recovery of enzymic activity, the reaction with gluten could not be reversed with such success. It is presumed that either hydroxylamine itself, or the work up of the reaction has a deleterious effect on the properties of gluten.

The reaction of gluten with potassium cyanate

At pH 8, approximately 75 p. 100 of lysine residues in gluten are modified in two hours (Table 3). This modified gluten had a mixogram with a reduced peak height and an increased mixing time, but the control sample showed no change in mixing properties (Fig. 6). Gluten which had been reacted for shorter times with

TABLE 3

Extent of modification of lysine residues in gluten with potassium cyanate

Reaction time	Amount of lysine modified (p. 100)
30 min.	33
60 min.	54
120 min.	86

potassium cyanate contained proportionately lower amounts of modified lysine, and the changes in the mixing properties were less marked than in the more extensively modified samples.

All of the gluten samples modified with potassium cyanate showed complete loss of baking properties. Microloaves baked with the cyanate-modified proteins did not rise during proofing and baking. This need not have arisen directly from the effect of the modified lysine residues. It is possible that there remained in the treated gluten, a small amount of free potassium cyanate which would kill the yeast cells in the dough. Microloaves baked from the control gluten showed no change in loaf volume from the untreated gluten.

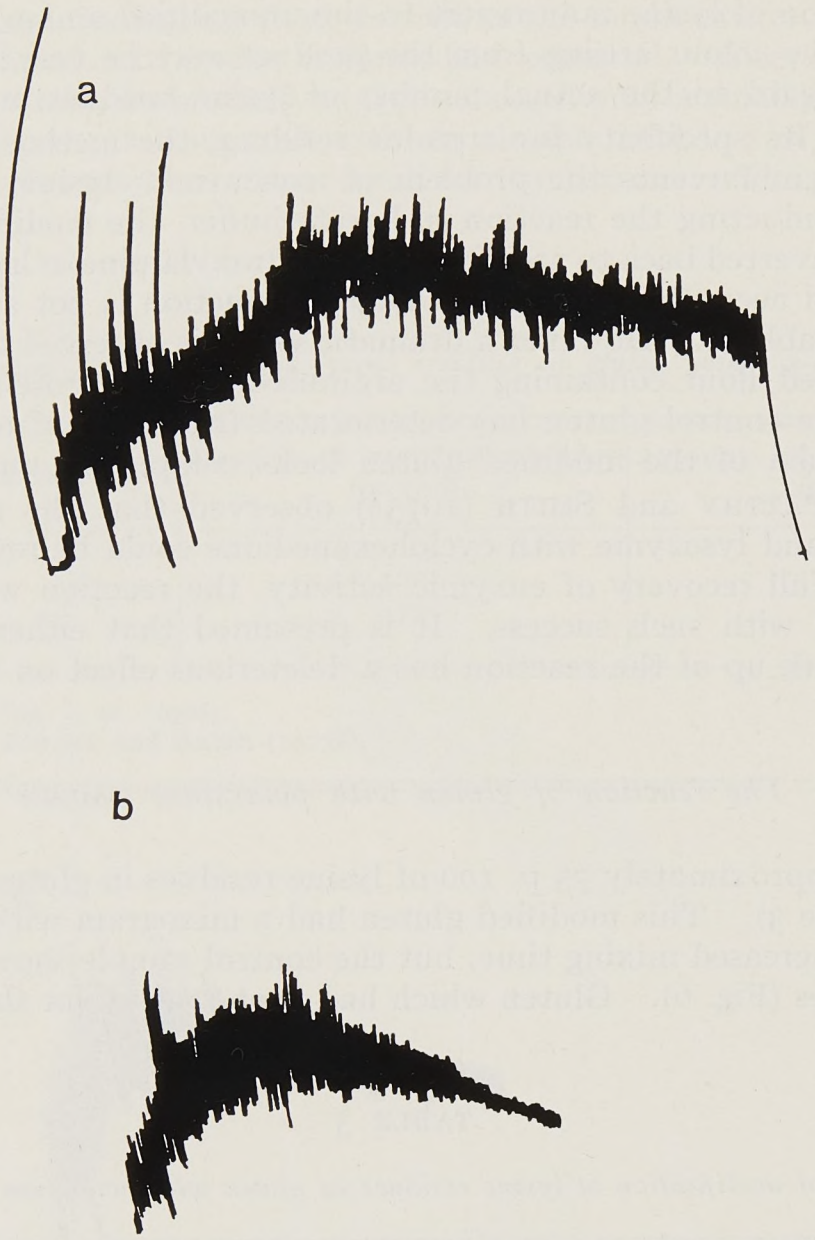

FIG. 6. — *Mixograms of reconstituted flours containing 29.75 g starch and (a) 5.25 g „ control " gluten for potassium cyanate modification; (b) 5.4 g potassium cyanate modified gluten.*

The reaction of gluten with N-acetylimidazole

Tyrosine groups in gluten are modified slowly by N-acetylimidazole under non-denaturing conditions in pH 8 buffer (Table 4). When the reaction was carried out in 3 M urea at pH 8, the tyrosine groups were found to react much more quickly.

TABLE 4

Extent of modification of tyrosine residues in gluten with N-acetylimidazole

Reaction conditions	Amount of tyrosine modified (p. 100)
pH 8, no urea	12
pH 8, 3 M urea	85

In two hours in 3 M urea, the reduction in absorbance at 280 nm represented 85 p. 100 of the tyrosine residues present. This suggested that the tyrosine groups are mostly buried in the protein molecule and are exposed only when the protein is denatured by urea.

Discussion

The chemical modification of amino acid residues in gluten has revealed that in general the conformation of the protein is similar to that of other proteins. The charged residues, lysine and arginine, can be modified extensively with relative ease, thus indicating that they are in accessible positions on the surface of the molecule. The glutens so modified show a marked deterioration in their mixing and baking properties. It seems unlikely, however, that the proteins are denatured in the reaction mixture prior to modification since the results of the control experiments, where the gluten was subjected to the experimental conditions without the reagent being present, show little change in the properties of the proteins.

The reaction of tyrosine residues with N-acetylimidazole contrasts with the ease of modification of lysine and arginine. In pH 8 buffer under non-denaturing conditions, there was a low reactivity of the tyrosine groups towards this reagent. This observation could have been due to either the lack of solubility of gluten at pH 8, or to the inaccessibility of the tyrosines in buried sites. When the reaction was carried out in 3 M urea buffered at pH 8, the tyrosines reacted much more extensively. The denaturing effect of this solvent apparently unfolded the protein to expose many more tyrosine residues. The solubility of gluten in 3 M urea is also greatly increased. However, it was noticeable that the lysine residues reacted extensively with potassium cyanate at pH 8 without the needs to increase the solubility by the use of urea. Similarly, the arginine residues reacted with 1,2-cyclohexanedione at pH 9, at which pH the gluten is not significantly more soluble than at pH 8. It appears more likely therefore, that the increased reactivity of tyrosine residues in 3 M urea is a result of the unfolding effect of the urea and not of the greater solubility of the protein. Tyrosine residues normally could be expected to react at least as fast as lysine and arginine residues if they were in exposed sites, and it therefore appears that they are buried within the structure of the protein.

The reaction of gluten with sodium hydroxide produced some interesting results. The molecular weight range of the alkali-treated gluten was unchanged from that of untreated gluten, despite the destruction of all disulphide groups in the protein. If gluten consisted of relatively small subunits held together by disulphide linkages, the molecular weight of the gluten should be drastically reduced by this treatment. That no such reduction in molecular weight range is observed is evidence that the disulphide groups do not form the major force holding small subunits together in larger aggregates. Hydrogen bonding, hydrophobic interactions and electrostatic attractions together must contribute the major forces determining the quaternary structure of the protein complex.

It is interesting to note that gliadin, which is presumed to be the low molecular weight fraction, is more rapidly attacked than glutenin. This observation in itself is suggestive of the absence of intermolecular disulphide groups, since an intermolecular disulphide should be more rapidly attacked than an intramolecular link. The cystine involved in an intermolecular link must be on the surface of each subunit involved. On the other hand, cystines of intramolecular

bridges need not be on the surface. Hence, if they were in the interior of the molecule they would be expected to react more slowly. The mechanism of attack by hydroxide requires at least one half of the cystine to be on the surface where it would react rapidly. Some of the disulphides of gliadin are evidently in such a position because of the rapidity with which they are destroyed. Having broken the disulphide bridge the molecule can unfold, thus exposing the other half of the cystine residue to further attack. In glutenin, the disulphides are resistant to hydroxide and are clearly in sites where they are protected from attack. It is in the glutenin fraction where intermolecular disulphides are postulated to exist, but the lack of reactivity of the disulphides is inconsistent with this hypothesis. It is assumed, therefore, that the attack by hydroxide on the cystine residues of glutenin occurs while the molecule is undergoing its natural unfolding and refolding.

The observation by BERNARDIN (1978) that the molecular weights of gliadin and glutenin are not very different indicates the necessity to discover more about the arrangements of amino acids in each type of protein. A more detailed picture of the structure would be revealed by observing the effects of chemical modification on purified, homogeneous gluten proteins. The information obtained by such a study would help in understanding the role each component of gluten plays in determining the properties of this complex protein.

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Résumé

Modification chimique comme moyen d'étude de la structure de gluten

« La réactivité chimique d'un acide aminé d'une protéine permet de mesurer son accessibilité, ce qui donne des indications sur sa localisation et son rôle dans la structure de la protéine native.

Les acides aminés basiques du gluten, lysine et arginine, sont respectivement profondément modifiés par le cyanate de potassium et le 1-2 cyclohexanedione.

Les résidus cystines de la gliadine réagissent très rapidement avec la soude; ceux de la gluténine sont beaucoup moins réactifs. La tyrosine ne réagit rapidement avec le N-acetylimidazole qu'en présence d'urée 3 M. Les résultats suggèrent que les liaisons disulfures ne sont pas le facteur principal de la taille des agrégats des sous-unités du gluten. »

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The role of disulfide bonds and their localisation in wheat protein molecules

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Summary

The reduction dynamics of S-S bonds contained in wheat glutenin and gliadin, isolated of flours of various baking quality, was carried out in the absence of urea (so called intermolecular ones) in order to demonstrate the distribution of bonds of different accessibility for the reducing agent in the mentioned protein fractions. In both fractions of two types the S-S bonds were demonstrated, the levels of which being differentiated in samples of various baking quality and showing some regularities. The possible functions of those S-S bond types are discussed and compared with the Ewart's (9) concepts of glutenin structure.

Key words : *disulfide bonds — localisation — wheat protein molecule.*

Introduction

During the period of sixties many scientific groups were intensively working on the contents and role of SH groups and S-S bonds in the structure of gluten complex. According to the results of Belderok's (1), Ewart's (8, 9), Bloksma's (5), Mauritzen's (11) and other groups it was suggested, that the SH group contents constituted only some percents of those of S-S bonds and that they influenced the lability of those bonds, particularly, when they were present as the low molecular weight SH compounds. It was also suggested that the contents of intermolecular S-S bonds (those, which can be determined without unfolding of polypeptide chain), as well as those of intramolecular, which are reducible only after the treatment with 7 M urea, should be connected somehow with the wheat baking quality. It was shown also, that the sulfur exchange rate between the SH groups and S-S bonds remains in the reverse relationship with the baking quality when the gluten is investigated in the gel form or as the component of dough. In spite of many investigations concerning those problems it was obtained until now no clear evidence on the role of S-S bonds in the building up of gluten complex and in the next period this problem became less interesting for the scientists.

We consider, that the main reason of the lowering of the interest in the S-S functions was based on the lack of proper determination methods of S-S bonds in cereal proteins, because the conditions of amperometric or dithisone methods (the oxido-reduction potential) could not be as low, as they should be (the full reduction could not occur). Just after the new methods were elaborated (9), which were based on the reduction of proteins using the stronger reducing agents (2-mercapto-ethanol, dithiothreitol), it could be possible to determine the levels of S-S bonds, which could be comparable with those of Cystein + Cystin, as determined in the amino acid analyser. It could be shown afterwards, that the level of total, as well as intermolecular S-S bonds demonstrated no relationship with wheat baking quality. It could be also earlier deduced, as the Cystein + Cystin level

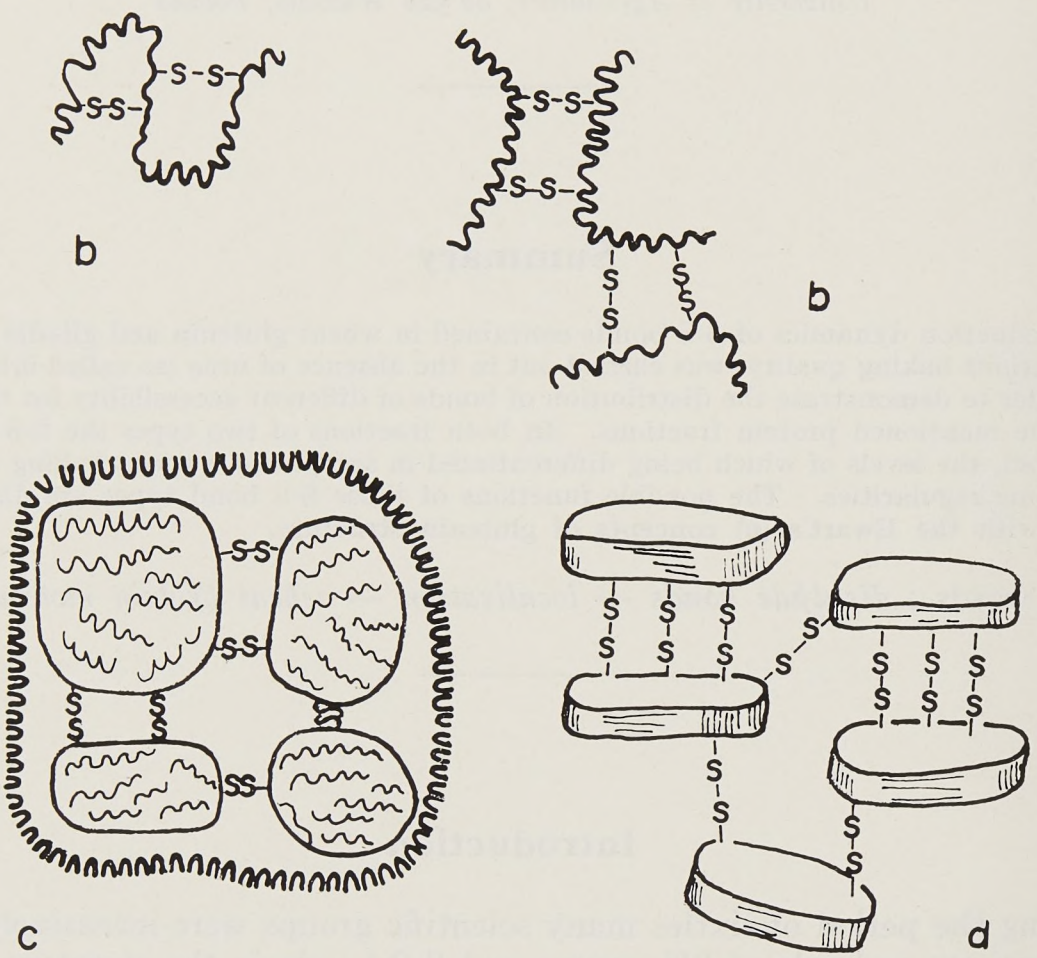

FIG. 1. — *The suggested different types of S-S bonds in wheat gluten proteins*; a) *intrachain*, b) *interchain*; c) *intermolecular*; d) *taking part in the gel formation.*

in different wheat varieties grown in normal conditions, was shown to be similar. On the other hand when we reexamined the experiments of Mauritzen (11) and those of Lee (10), concerning the exchange rate between the SH group and S-S bonds, we could not obtain the relationship between this value and the baking quality (2). But the difference between the experiments of above authors and those of ours was, that in the former the gluten was used in the gel form, or as the component of dough, whereas in the latter ones it was dispersed in acetic acid. It could be suggested therefore, that the demonstrated by the above authors reverse relationship between the exchange rate and baking quality, can concern only those S-S bonds, which occur in the gluten gel form or in dough but are lacking in the dispersed gluten.

We have already in 1975 demonstrated (4), that in dispersed gluten two types of intermolecular S-S bonds exist, which are differentiated by the accessibility for the reducing agent. This was demonstrated as the two-step reduction process, in which the following steps were divided each other by means of a break. Therefore we have suggested the existence in wheat gluten at least four types of S-S bonds, which are schematically expressed in the Figure 1. These are as follows:

a — intramolecular S-S bonds, which can be reduced only after the unfolding of polypeptide chain;
b — less accessible for the reducing agent intermolecular S-S bonds;
c — easily accessible intermolecular S-S bonds;
d — intermolecular S-S bonds, which occur only in gel form of gluten or as the component of dough, to bind together the separate molecules to form the giant protein aggregates.

In our further investigations, the results of which were until now not published, we determined the distribution of intermolecular S-S bonds of the types *b* and *c* in glutenin and gliadin by means of analysing the reduction dynamics of those bonds in the protein components mentioned above, which were isolated from flours of various baking quality.

Material and methods

Glutenin and gliadin used in experiments described below were obtained from glutens isolated of flours of differentiated baking quality by procedure of Coates and Simmonds (6), in which the defatting with butanol was omitted. The gluten dispersion of 0.05 M acetic acid was then separated in the Sephadex G 100 column on the preparative scale using 0.05 M acetic acid and the glutenin and gliadin peaks were collected separately and concentrated. 10 mg of proper protein in 1 cm^3 of acetic acid was reduced with 40 mm^3 dithiothreitol (9.2 mg of pure compound) dissolved in 0.05 M pyrophosphate during 2 till 32 hours at 25 °C and then the levels of SH groups were determined using the own method (3) based on the Ellman's reagent (7), i.e. 5,5'-dithio-bis-(2-nitrobenzoic) acid. Of the samples cited in this publication Saratowskaja and 694/74 showed high baking quality, but Tetrix and 1347/74 — low quality.

In experiments concerning the inheritance of S-S bond contents and distribution by the F_1 hybrids of winter wheat, the male sterile maternal forms (MS), the physiologically fitting to the former, paternal forms, possessing the fertility restoring ability (RS), as well as the crossing hybrids of F_1 generation, were used. Two series of experiments were carried out, in which the 4 and 14 combinations of samples were used. The grains were milled, flour extracted, as mentioned above (6) and in the acetic acid fraction the so called intermolecular and total S-S bond level determined using the own method (3).

Results and discussion

The results of experiments concerning the reduction dynamics of S-S bonds in glutenin and gliadin, demonstrated in Figure 2, as well as in tables 1 and 2 show, that in all investigated samples of glutenin and gliadin the reduction of inter

TABLE 1

The reduction dynamics of intermolecular S-S bonds (accessible for the reducing agent in the absence of urea) in glutenin (peak I) and gliadin (peak II), the reducing conditions as described in the methods (values given in SM uaeq/lg of protein).

Wheat variety	Peak	Reducing time 2	3	4	5	6	8	10	12	14	16	18	24	32	36	48	Level of S-S bonds reduced in p. 100 of totaly reduced
1347/74	I	42.5	—	49.0	*64.0*	*66.5*	*65.5*	*68.5*	91.0	—	—	—	109.0	—	—	—	60.1
	II	44.0	—	61.0	—	60.0	*90.0*	*97.0*	*92.0*	—	*99.0*	—	181.0	—	—	—	52.5
Tetrix	I	54.0	63.5	58.0	*80.0*	*88.0*	*79.0*	*80.5*	*91.0*	—	—	—	110.0	—	—	—	72.7
	II	44.0	—	52.0	*110.0*	*113.5*	*98.0*	*114.0*	*101.0*	—	124.0	138.0	180.0	176.5	—	—	62.7
694/74	I	25.5	*36.0*	*43.0*	—	*44.0*	*46.0*	*46.5*	*77.5*	—	—	—	84.0	—	—	85.5	53.5
	II	12.0	—	25.0	—	—	59.5	*97.5*	*105.0*	*90.0*	*115.0*	—	*114.0*	—	145.5	176.0	65.3
Saratowska	I	23.0	—	*49.0*	*56.5*	*54.0*	81.0	—	—	—	—	—	104.5	—	—	—	52.3
	II	25.0	—	46.5	—	—	119.0	*140.0*	*130.0*	*135.0*	*135.0*	—	166.0	202.5	205.5	—	65.8

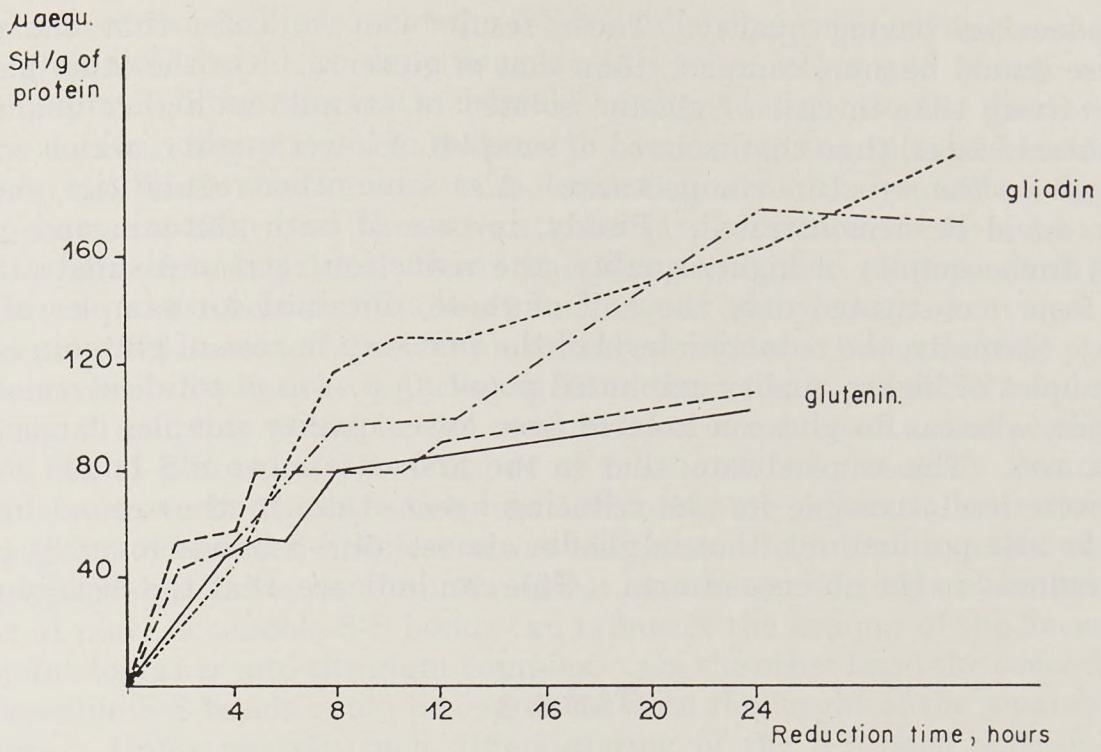

FIG. 2. — *Reduction dynamics of intermolecular S-S bonds in glutenin and gliadin of Saratowskaja and Tetrix.*

——— ····· Saratowskaja.
— — — —·—·— Tetrix.

molecular S-S bonds proceeded in two distinguished steps. Also in this case, similarly to that of gluten complex (4), the following reduction steps were separated by an break. This break was starting in case of glutenin earlier (after 4-5 hours), than in that of gliadin (after 5-10 hours), but this starting time seems to be

TABLE 2

Some characteristic data of the reduction dynamics of S-S bonds reduced in the absence of urea

Breack starting time, h.		The level of first step	The lenght of break	p. 100 of reduction in the I step	Final reduction level	The SH level after 2 hours
Glutenin st. . .	4	44	4-10/6	53.5	85	25
Glutenin st. . .	4	53	4- 6/2	52.3	104	23
Glutenin f. . .	5	66	5-10/5	60.1	109	42
Glutenin f. . .	5	80	5-10/5	72.0	110	54
Gliadin st. . . .	10	97	10-14/5	65.3	176	12
Gliadin st. . . .	10	137	10-16/6	65.8	205	25
Gliadin f. . . .	8	95	8-16/8	52.5	181	44
Gliadin f. . . .	5	107	5-12/7	62.7	180	44

Samples of : st — high and f — low quality.

independent on baking quality. Those results can indicate, that the gliadin structure should be more compact, than that of glutenin. On the other hand the break starting time in case of gliadin isolated of samples of higher quality was demonstrated later, than that isolated of samples of lower quality, which could be dependent on the structure compactness. Also some other regularities, shown in table 2, could be demonstrated. Firstly, in case of both glutenin and gliadin, isolated from samples of higher quality, the reduction rates measured after the second hour, constituted only the half of those, obtained for samples of lower quality. Secondly, the reduction level of the first step in case of glutenin isolated from samples of higher quality amounted about 53 p. 100 of total intermolecular S-S bonds, whereas for glutenin isolated from lower quality samples it reached 60 or 73 p. 100. This can indicate, that in the first case more S-S bonds occured, which were less accessible for the reducing agent, than in the second one. It should be also pointed out, that in gliadin almost all S-S bonds (over 85 p. 100) can be reduced in the absence of urea. This can indicate, that the distribution of

TABLE 3

The average levels of intermolecular and total S-S bonds and the p. 100 contents of the former as related to total ones in uaequivalents of SH /1 g of protein.

Type of bonds	Form	1974	1975
Total S-S bonds	MS	161.7	169.0
	RS	160.0	173.0
	F_1	170.7	172.0
Intermolecular S-S bonds	MS	92.7	100.2
	RS	104.6	98.2
	F_1	88.5	106.7
P. 100 of intermolecular as related to total S-S bonds	MS	55.9	59.2
	RS	65.6	56.6
	F_1	52.4	62.1
Number of combination		4	14

only easily reducible S-S bonds are of significance in the formation of baking quality, as concerns this protein fraction. This last indication could be contrary to the relations occuring in glutenin. The last question is, whether the lenght of the break between the first and second reduction steps is connected somehow with the difference in the strenght of binding represented by S-S bonds reduced in both steps. This should be most interesting particularly in case of gliadin, for which the break is much longer, when isolated from samples of lower quality, than for other ones.

We wanted simultanously to demonstrate the possibility of the inheritance of some structural features connected with the S-S bonds. The results of experiments concerning the S-S bond contents in gluten for two series are given in table 3, as their averages. It can be concluded from these data, that neither the total contents of S-S bonds, nor those of intermolecular ones, could be inherited of any parental form by the F_1 hybrids. However the percent of intermolecular S-S bonds, as related to their total contents, seems to be inherited of the maternal form.

This statement can indicate, that the S-S bond distribution in whole gluten (maybe also in glutenin) should be depending on this form rather and bound with the sample quality. Thus this feature should be characteristic for the wheat variety.

The newer suggestions of Ewart (9) concerning the glutenin building up anticipate the linear structure of its molecules, in which the separate polypeptide chains, posessing their own characteristic tertiary structure, are bound by means of S-S bonds, forming long, linear complex molecules. Independently those linear structures are bound with other similar structure by means of covalent bonds, including the S-S ones, to form complex giant molecules. It seems quite probable, that the accessibility for the reducing agent of those two types of S-S bonds should not be equal and this would be the confirmation of our results, concerning glutenin and initial gluten. Of two types of S-S bonds suggested by Ewart, more accessible seem to be those, which join each other the separate linear structures, to form giant complexes, and less accessible those, which bind together the separate polypeptide chains, to form the linear structures. In this assumption, the content of more accessible S-S bonds can influence the amount of the linear structures, joint together into the giant complex. On the other hand the content of the less accessible S-S bonds could be connected with the lenght of the separate linear structures. Unfortunately such differentiation of the S-S bond accessibility in case of gliadin can be at present without any elucidation.

Conclusions

1. — In glutenin, as well as in gliadin two types of S-S bonds reducible in the absence of urea exist, which are differentiated by the accessibility for the reducing agent.

2. — The reducing rate of the more accessible S-S bonds, as measured close to the start, is reversly related to the sample quality in both glutenin and gliadin, which is connected with the molecule compactness.

3. — The level of more accessible S-S bonds in glutenin is lower in samples of high quality, than in those of poor quality.

4. — The distribution of S-S bonds in glutenin of various quality is not uniform and influences this property.

5. — The distribution of S-S bonds in gluten (the p. 100 of intermolecular ones, as compared to total ones), but not the general amounts, can be inherited from maternal forms by the F_1 hybrids.

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Résumé

Rôle et localisation des ponts disulfures dans les molécules protéiques du blé

Il y a déjà 5 ans, nous avons commencé nos travaux sur la cinétique de réduction des ponts disulfures intermoléculaires dans les protéines du gluten de blé. Nous avons pu observer que pendant le processus de la réduction, une rupture apparait dans l'augmentation du nombre des groupes SH. C'est pourquoi nous avons pensé qu'il existe dans le complexe de protéines deux types de ponts disulfures intermoléculaires qui diffèrent dans leur degré d'accessibilité à l'agent réducteur.

Dans la suite des recherches, nous avons étudié ces cinétiques de réduction des ponts disulfures en absence d'urée dans les gluténines et gliadines, qui étaient isolées de farines de différentes qualités boulangères. Les résultats démontrent que pour les deux types de protéines, la réduction des ponts disulfures se poursuit bien en deux étapes, qui se distinguent par l'écart des degrés de stabilité du groupe SH.

Cette rupture commence plus tôt pour la gluténine que pour la gliadine mais ce point ne semble pas être en rapport avec la qualité.

D'un autre côté, dans le cas de la gliadine, le moment où cette rupture commence, est plus tardif pour des échantillons de qualité supérieure que pour des échantillons de qualité inférieure. On pourrait également dans le cas de la gluténine, mettre en relation des différences de qualité avec le pourcentage des ponts disulfures les plus accessibles. Enfin, l'intervalle entre les deux étapes de la réduction est inférieure dans le cas de la gluténine à ce qu'il est dans celui de la gliadine.

Les résultats ci-dessus suggèrent que la distribution des ponts disulfures dans le gluten des différents échantillons étudiés n'est pas uniforme et peut jouer un rôle très important dans la compacité des protéines. Par conséquent, la relation entre les ponts disulfures facilement ou difficilement accessibles réduits en absence d'urée, pourrait être d'importance pour le mécanisme des propriétés rhéologiques de la pâte.

Nous débattrons également dans cet exposé les concepts plus récents concernant la signification des ponts disulfures pour la formation de la structure du gluten de blé et pour la qualité de la farine.

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Interaction between lipids and gluten proteins

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Summary

Free wheat flour lipids interact with gluten proteins during dough mixing or gluten washing. The role of that interaction in breadmaking potential and performance has been studied, and the results have been used to postulate model systems. Lipids may mediate protein-protein interactions and affect physico-chemical properties of the interacting components. Whereas lipid-protein interactions govern dough properties, lipid complexing with starch is significant in the baking process. Insight into some of the changes that take place can be gained from microscopic studies. The information that has been obtained in studies on lipid-gluten interaction is used to explain changes in breadmaking. The practical implications of the various interactions in the production of protein-enriched bread are described.

Key words : *lipids — gluten — interactions — flour — baking quality.*

The significance of interactions between lipids and gluten protein has been studied little because of the complexity of the system and the paucity of methods for the study of such interactions (POMERANZ, 1973). This review summarizes work, mainly from the author's laboratories, on lipid-gluten interaction as it relates to breadmaking.

Interaction in gluten washing or dough mixing

In washing out gluten or in dough making, one-third to two-thirds of the wheat flour free lipids (some free nonpolar and practically all polar components) are bound and subsequently unextractable with petroleum ether (CHIU and POMERANZ, 1966; MECHAM, 1971). Extractability of lipids depends on several factors that include particle size, composition and age of the wheat flour, dough composition (including amounts and types of flour and added lipids), water content of extractants and of flour lipids, presence of shortening and /or surfactants, work input and atmosphere in dough mixing, and stage in bread production (MECHAM,

1971; POMERANZ, 1971; CHUNG and POMERANZ, 1977; CHUNG *et al.*, 1978; MORRISON, 1978; POMERANZ, 1980; POMERANZ and CHUNG, 1980). Starch lipids can be excluded from consideration of binding during dough mixing because starch lipids are bound very tightly as inclusion compounds with amylose, and consequently are not available for reacting with gluten proteins (ACKER *et al.*, 1968).

Available information indicates that in dough, polar lipids (glycolipids and phospholipids) interact mainly with gluten rather than with the soluble wheat flour proteins. This is of significance as gluten is the skeleton or framework of wheat flour dough and is responsable for gas retention that is required in the production of light, yeast-leavened products. In the baked bread, much of the interaction is with starch. This is of significance as the starch governs, to a large extent, freshness retention of the baked bread.

Model systems

Methods of study, bonds, and mechanisms of interaction between lipids and proteins and starch are summarized in Table I (from WEHRLI, 1969), and some of the proposed models are shown in Figure 1. Almost a quarter century ago, HESS (1954) proposed, on the basis of X-ray, electron microscope, and optical measurements, a structural relationship of proteins, lipids, and starch in wheat flour, in which wedge protein deposits are surrounded by a lipid layer, beyond which lie adhesive layers and still further starch granules. HESS and MAHL (1954), proposed a model (Fig. 1*a*) in which the adhesive protein is bound to starch through a lecithin layer.

In 1957 TRAUB *et al.* (1957) reported that a 46-Å spacing observed in X-ray studies of wheat and flour was due to phospholipids associated with protein fibers

TABLE I

Methods of study bonds, and mechanisms of interaction between lipids, proteins and starch

Method of Study	Type of bond between glycolipid and:		
	Starch	Gliadin	Glutenin
Solvent extraction of gluten proteins		Hydrogen	Hydrophobic
Lipid binding in starch dough	Hydrogen	—	—
Infrared	Hydrogen	Van der Waals, hydrogen	Van der Waals, hydrogen
NMR	Hydrogen, some induced dipole	—	Hydrophobic and hydrogen
Autoradiography	Strong in bread	—	In dough
Baking test	Hydrophobic and hydrogen essential		

in the form of bimolecular leaflets. Then GROSSKREUTZ (1961) studied the structure of wheat gluten by electron microscopy and X-ray techniques and proposed a lipoprotein model involving a bimolecular lipid layer structure (the Danielli and Dawsontype membrane) as shown in Figure 1 (*b*). Grosskreutz showed that the proteins in moist gluten consist of folded, polypeptide chains in the α-helix configuration, arranged into flat platelets of the order of 70 Å thick. Extraction of the phospholipids did not affect the basic platelets but seriously impaired their abi-

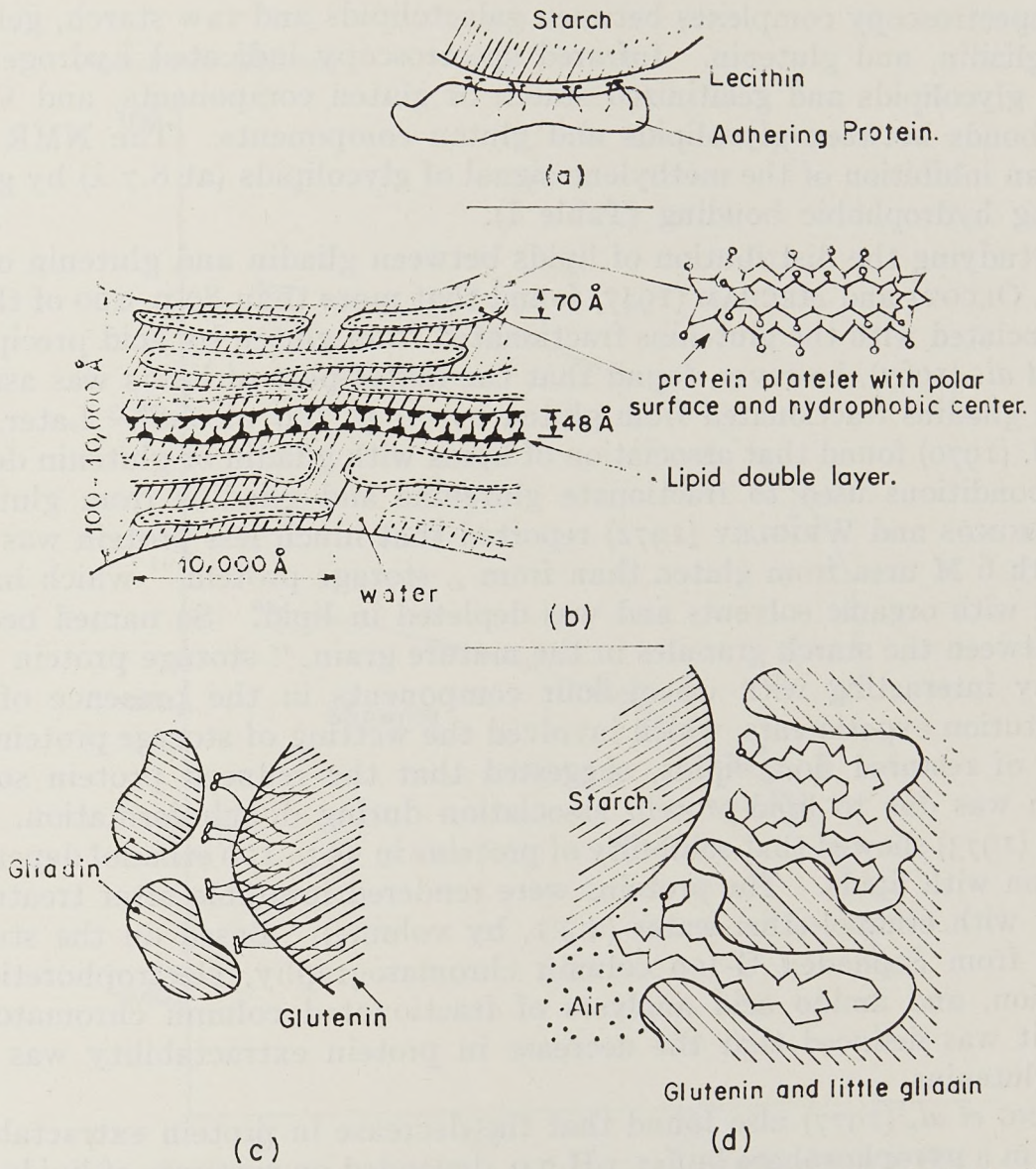

FIG. 1. — *Proposed models of the complex formed in breadmaking*: (*a*) *Starch-lipid-adhesive protein complex in flour by* HESS *and* MAHL (1954); (*b*) *Lipoprotein model by* GROSSKREUTZ (*1951*); (*c*) *Gliadin-glycolipid-glutenin complex by* HOSENEY *et al.* (*1970*); (*d*) *Starch-glycolipid-gluten complex by* WEHRLI (*1969*).

lity to bond into sheets capable of sustaining large plastic deformation. From X-ray evidence of the phospholipid structure in gluten, GROSSKREUTZ (1961) deduced *a*) that proteins formed well oriented bimolecular leaflets of the type found in myelin, *b*) that lipoprotein occupies about 2 to 5 p. 100 of the elastic gluten structure, and *c*) that protein chains are bound to the outer edge of aphospholipid bimolecular leaflet array (probably via salt-type linkage between acidic groups of the phospholipid and the basic protein groups).

Hoseney *et al.* (1970) found that free polar lipids (principally glycolipids) are bound to the gliadin proteins by hydrophilic bonds and to the glutenin proteins by hydrophobic bonds, as it is shown in Figure 1 (*c*). In unfractionated gluten, the lipid apparently is bound to both protein groups at the same time. The simultaneous binding of polar lipids to both gliadin and glutenin may contribute to the gasretaining ability of gluten.

Wehrli and Pomeranz (1970*a*) supported the model proposed by Hoseney *et al.* (1970) after studying the interaction of galactolipids with wheat flour macromolecules. They investigated by infrared and nuclear magnetic resonance (NMR) spectroscopy complexes between galactolipids and raw starch, gelatinized starch, gliadin, and glutenin. Infrared spectroscopy indicated hydrogen bonds between glycolipids and gelatinized starch or gluten components, and Van der Waals bonds between glycolipids and gluten components. The NMR spectra showed an inhibition of the methylene signal of glycolipids (at 8.7 λ) by glutenin, indicating hydrophobic bonding (Table I).

In studying the distribution of lipids between gliadin and glutenin of wheat proteins, Olcott and Mecham (1947) found that more than 80 p. 100 of the lipids were associated with the glutenins fractionated from gluten by acid precipitation. Ponte *et al.* (1967), however, found that the major part of lipids was associated with the gliadins fractionated from gluten by 70 p. 100 alcohol. Later, Hoseney *et al.* (1970) found that association of lipids with gliadin or glutenin depended on the conditions used to fractionate glutenins and gliadins from gluten.

Simmonds and Wrigley (1972) reported that much less protein was extractable with 6 M urea from gluten than from „ storage protein " which had been prepared with organic solvents and was depleted in lipid. So named because it exists between the starch granules in the mature grain, " storage protein " forms gluten by interacting with wheat-flour components in the presence of water. Reconstitution experiments, which involved the wetting of storage proteins in the presence of restored flour lipids, suggested that the reduced protein solubility in gluten was due to lipid-protein association during dough formation. Charbonnier (1973) showed that solubility of proteins in 55 p. 100 ethanol depended on interaction with lipids. The proteins were rendered insoluble after treatment of the flour with ethanol-ether-water (2:2:1, by volume). Based on the studies of fractions from Sephadex G-100 column chromatography, electrophoretic characterization, and amino acid analyses of fractionated column chromatography eluates, it was deduced that the decrease in protein extractability was mainly due to glutenins.

Chung *et al.* (1977) also found that the decrease in protein extractability in 3 M urea in a pyrophosphate buffer, pH 7.0, depended on amounts of lipids removed from a poor breadmaking quality flour (Chiefkan/Tenmarq — Cfk/Tm, KS 501097) and from a good quality flour (Shawnee); protein extractability was determined in flours that were previously defatted with Skelly B, benzene, acetone, and 2-propanol (Fig. 2.) The amount of lipids extracted during defatting ranged from 0.88 to 1.41 p. 100 for Cfk/Tm and 1.05 to 1.47 p. 100 for Shawnee flour (least with Skelly B, next with benzene, and most with 2-propanol). The increase in total extractable lipids was primarily due to an increase in extracted polar lipids. Regardless of lipid contents in flour, more proteins were extracted from the poor than from the good quality flour. Similarly, the absorbance of protein extracts of Shawnee flour in 0.01 M sodium pyrophosphate (Na-pp), 0.005 N and 0.05 N acetic acid (AcOH) decreased curvilinearly with increasing amounts of lipids removed from flour by Skelly B, benzene, acetone, and 2-propanol (dotted

lines in Fig. 3). Reconstituting the defatted flours with the lipids extracted by the various solvents restored protein solubility (solid lines in Fig. 3), but to different extents depending on the lipid extractant (Skelly B, benzene, acetone and 2-propanol) and protein extractant (0.01M Na-pp buffer, 0.005N and 0.05N AcOH). The solubility of non-gluten proteins (soluble in Na-pp) was completely restored by lipid reconstitution except for the 2-propanol treatment. Protein solubility in 0.005N AcOH was restored completely by lipid addition for Skelly B and benzene treatment but only partially for the acetone and 2-propanol treatments. For

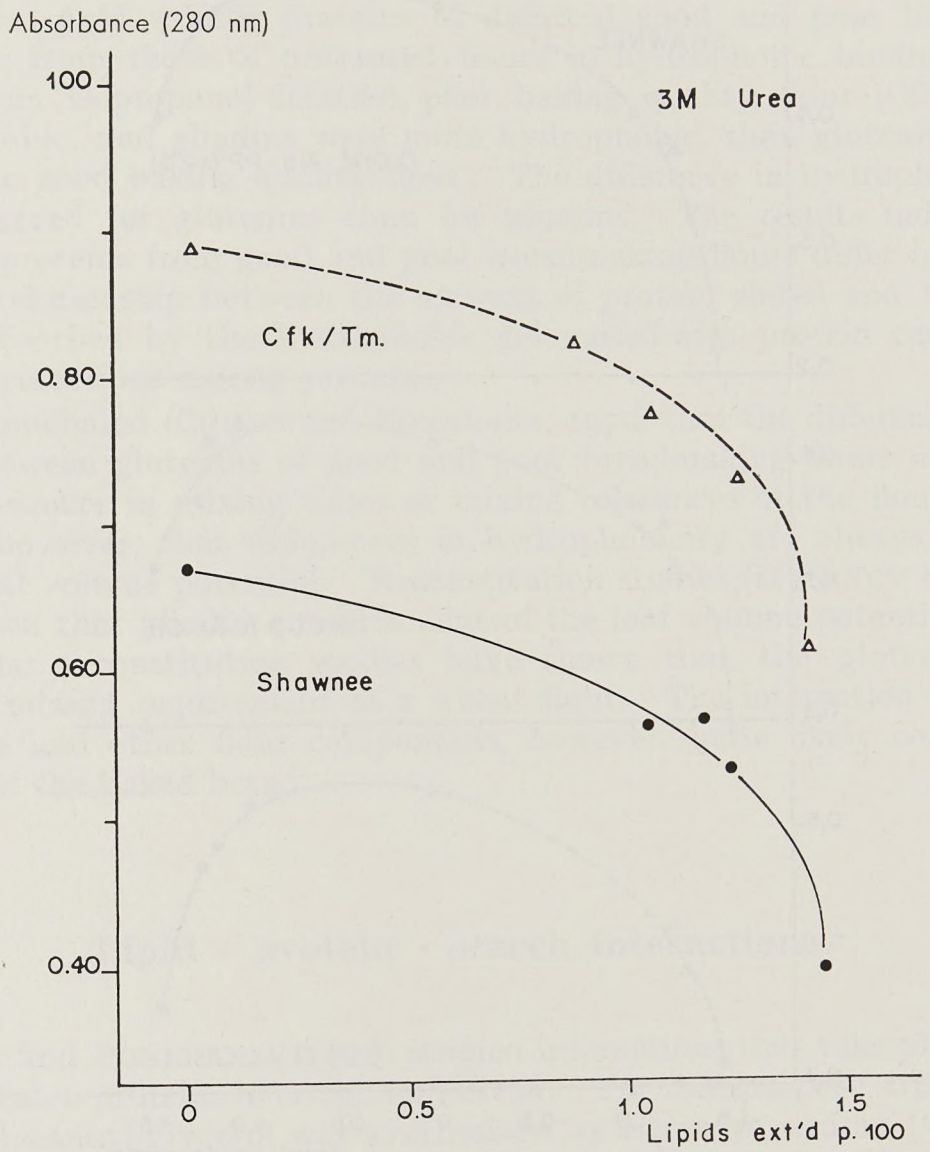

FIG. 2. — *Solubility of proteins (as determined by absorbance at 280 nm of 3 M urea extracts) in Shawnee and KS 501097 (Cfk/Tm) flours from which were extracted various amounts of lipids* (CHUNG *et al.*, *1977*).

0.05N AcOH-soluble proteins, presumably gluten proteins, restoration of protein solubility was complete only for Skelly treatment which involved free lipids only. The restoration was almost irreversible with 2-propanol treatment, which involved bound as well as free lipids. Only nonpolar lipids showed solubilizing effects on proteins of both poor and good quality flours; polar lipids had no solubilizing effects or a slight insolubilizing effect (protein aggregation) for good flour when they were separately reconstituted with defatted flours by blending in the dry state.

CHUNG and POMERANZ (1978) reexamined chemical interactions among proteins and between proteins and lipids. Proteins were extracted with 0.05N acetic acid from untreated and defatted flours, and the extracts were dialyzed, lyophilized, and fractionated by gel filtration on Sephadex G-100. The extracts and the fractions were characterized by starch and SDS polyacrylamide gel electrophoresis. Both the untreated and the defatted poor (Cfk/Tm) flours showed higher protein

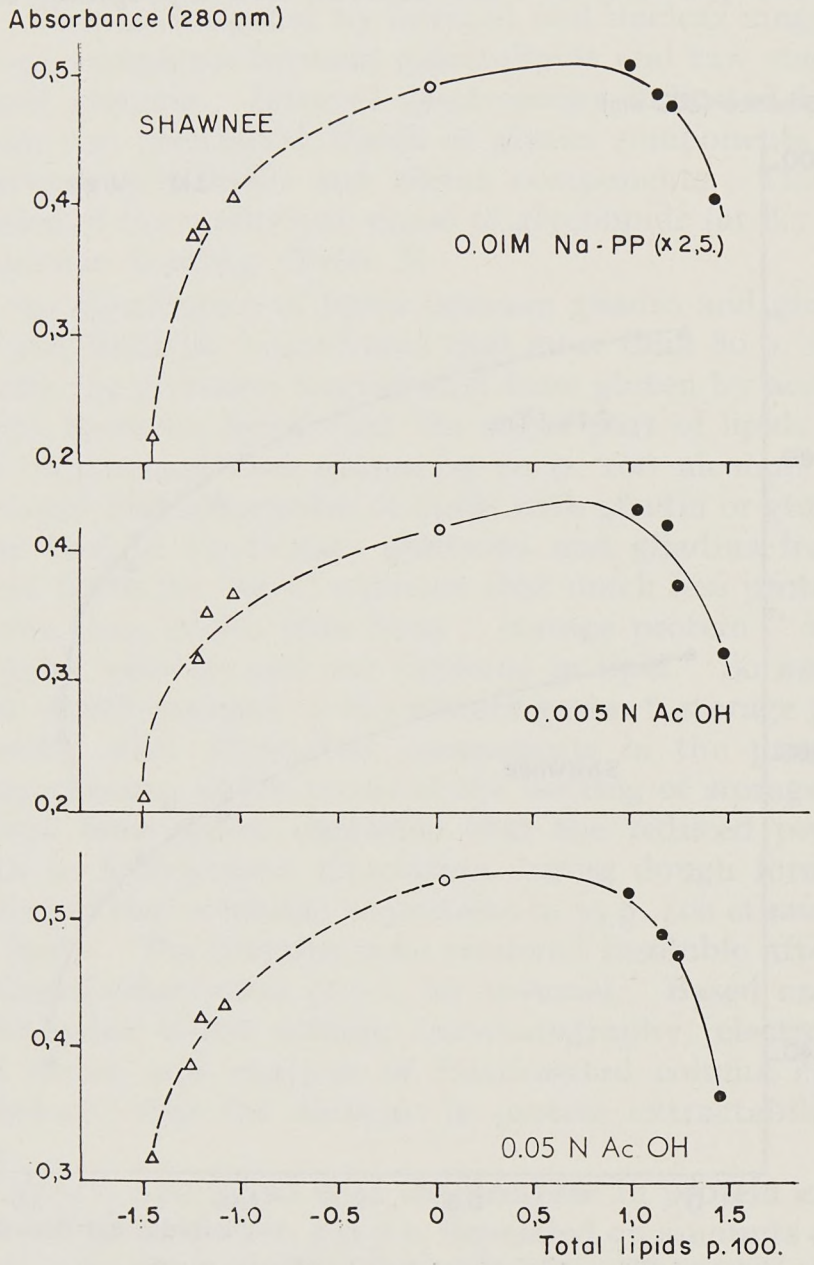

FIG. 3. — *Solubility of proteins (as determined by absorbance at 280 nm of 0.01 M Na-pyrophosphate, 0.005 N acetic acid, and 0.05 N acetic acid) in untreated, defatted (removed), and defatted-reconstituted (added) Shawnee flours* (CHUNG *et al.*, 1977).

extractability than did the good (Shawnee) flours. More proteins were extracted from untreated good and poor flours than from the corresponding defatted flours.

In elution profiles from the gel column, all protein extracts showed similar retention times for the glutenin, gliadin, and water soluble fractions. Quantitatively, the glutenins were the most variable among the gel-separated fractions in the acetic acid extracts from the flours. Whereas concentrations of gliadins were similar in all extracts, concentrations of glutenins depended upon the flour

samples, quantity and quality of lipids present in flour, and possibly defatting-solvent. The removal of flour lipids decreased glutenin: gliadin ratios. Glutenin and gliadin fractions differed between good add poor quality flours, and between untreated and defatted flours in number and intensity of electrophoretic bands.

In a subsequent study by CHUNG and POMERANZ (1979), the binding of acid soluble proteins from untreated and defatted good (Shawnee) and poor (Cfk/Tm) baking quality flours to Phenyl-Sepharose CL-4B was examined by batch— and columnelution techniques. When a mixture consisting of hydrophobic gel and acid soluble flour proteins was eluted batchwise with five solvents, total absorption at 280 nm of the eluates was higher for the poor than for the good baking quality flour. Acid soluble proteins of defatted good and poor baking flours differed little from those of untreated flours in hydrophobic binding capacity. Glutenins from isopropanol-defatted poor baking quality flour (Cfk/Tm) were less hydrophobic, and gliadins were more hydrophobic, than glutenins and gliadins from the good baking quality flour. The difference in hydrophobicity was more pronounced for glutenins than for gliadins. The results indicated that acid-soluble proteins from good and poor breadmaking flours differ in hydrophobicity. The relationship between the amount of protein eluted and the amount of protein absorbed by the hydrophobic gel varied with protein concentration, indicating interactions among proteins.

It was concluded (CHUNG and POMERANZ, 1979) that the difference in hydrophobicity between glutenins of good and poor breadmaking flours may explain, in part, differences in mixing times or mixing tolerances of the flours. It does not follow, however, that differences in hydrophobicity are always causatively related to loaf volume potential. Reconstitution studies (HOSENEY *et al.*, 1969*a*, *b*) have shown that gliadin proteins control the loaf volume potential of wheat flour. Similar reconstitution studies have shown that the glutenin fraction governs the mixing requirement of a wheat flour. The interaction of glutenins with gliadins and other flour components, however, quite likely contributes to the quality of the baked bread.

Lipid - protein - starch interactions

WEHRLI and POMERANZ (1970*b*) studied interactions that take place between starch and gluten proteins in dough and bread. For that purpose, tritium-labeled galactosyldidecanoyl-glycerol was synthesized by a new procedure (WEHRLI and POMERANZ, 1969). Sections prepared from dough and bread containing the labeled galactolipid were studied by autoradiography. In the dough, the galactolipid was distributed in the gluten and, to a limited extent, in the starch; in the bread most of the galactolipid was in gelatinized (by oven heat) starch granules and formed a complex which seemed to be responsible for the improved retention of freshness in bread baked with glycolipids. WEHRLI (1969) proposed that glycolipids improved gas retention and, hence, loaf volume, by sealing the gas cells, presumably by complexing between the swelling starch and the coagulating proteins, as shown in Figure 1 (*d*).

DE STEFANIS *et al.* (1977) recently drew a conclusion similar to the one drawn by WEHRLI and POMERANZ (1970*b*) that the lipids bound to proteins during dough mixing are translocated and bound to starch during the baking. They found

that at the sponge stage, binding between added surfactants and the major flour components was not extensive. The additives were firmly bound to the gluten proteins during dough mixing and strongly bound to the starch by complexing with both amylose and amylopectin fractions in bread. They concluded, after studying model systems, that two concurrent phenomena occurred during baking: (*a*) the bonds between the gluten proteins and the additives weakened progressively (protein denaturation) as the dough temperature increased; and (*b*) as starch gelatinized above 50 °C, the additives, weakly bonded to proteins, readily formed a strong complex with starch and could thus be translocated from proteins to starch. In addition to the additives, triglycerides, free fatty acids, and lysophosphatidyl choline were also bound to the starch.

Seeing is believing

The structure of wheat-flour doughs and the development of the doughs into bread have been studied by the use of transmission electron (TEM) and light (LM) microscopy by SIMMONDS (1972) and by KHOO *et al.* (1975).

The effect of mixing on structure of doughs made from good and poor quality flours was studied by BECHTEL *et al.* (1978). They used LM and TEM to: *a*) elucidate microscopic changes associated with undermixed, optimally mixed, and overmixed flour-water doughs; *b*) determine differences among doughs formed from poor and good quality flours; and *c*) examine the ultrastructural changes that take place in a dough containing all bread ingredients during mixing, fermentation, proofing, and baking.

An optimally mixed dough formed from a good breadmaking composite flour had an even and continuous distribution of protein around starch granules and an even distribution of inclusions within the protein. The undermixed composite flour dough failed to have those characteristics. In the overmixed dough, there was an even distribution of inclusions within the protein, but the protein was not continuous. Protein strands were broken and the protein contained many large vacuoles. The vacuoles, apparently, weakened the protein by producing localized thin protein strands that were disrupted easily. Those results agreed with the findings of BAKER and MIZE (1946) who measured (by changes in dough density) vacuole formation in doughs and effects of vacuole formation on bread quality. Doughs that are inadvertently overmixed can be relaxed and then remixed to form an optimally mixed dough. We found that when an overmixed dough was allowed to relax, the protein vacuoles decreased in size and number (BECHTEL *et al.*, 1978). Possibly the relaxation and gentle remixing " mended " the broken protein strands so that an optimally mixed dough was obtained.

The poor quality flour dough differed strikingly from the good-quality flour doughs. The protein strands of the poor flour dough broke easily, even before the protein inclusions were uniformly distributed. In good quality flour, the tensile strength of the hydrated proteins apparently governs the structure of the dough and the bread. That was quite apparent when a good quality flour was grossly overmixed: the protein strands remained continuous with relatively few being broken. This " stability " to overmixing (and even undermixing) is one of the most important and desirable characteristics of good-quality flours. Fermentation of a complete dough produced gas vacuoles. After oven spring, protein strands

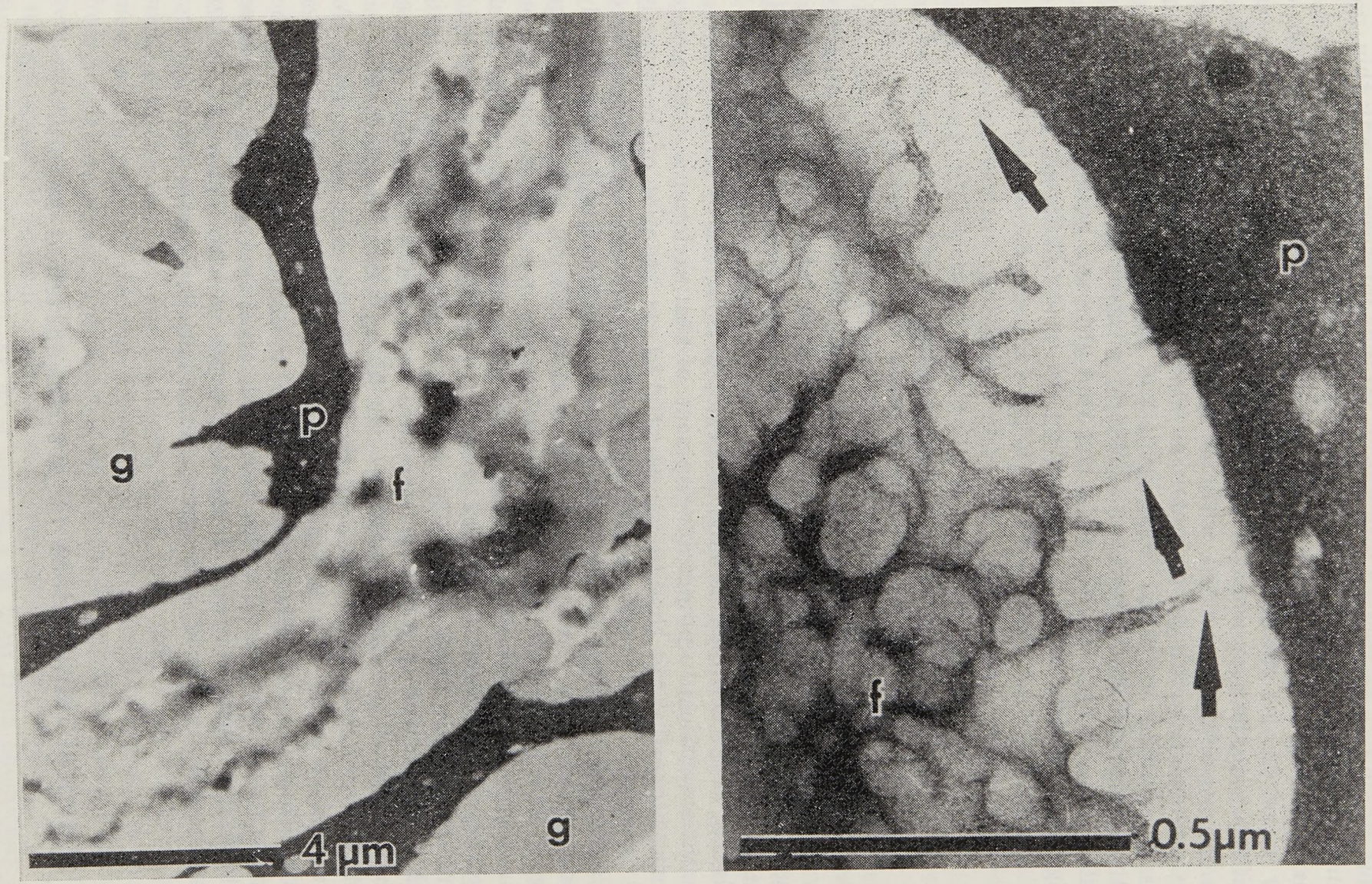

FIG. 4. — *(Left). TEM of the top center of a loaf immediately after baking. Note gelatinized starch (f) between thin protein strands. Note lack of vacuoles in protein (p); gas vacuoles (g). (Right). TEM of the protein (p)-gelatinized starch (f) interface in bread, highly magnified. Note fine connection (interaction, µ) between starch and protein strands (arrows).* (BECHTEL *et al.*, *1978*).

were thin and had small vacuoles. Starch granules in the bottom center of a loaf varied widely in degree of gelatinization after oven spring; starch started to gelatinize from the interior of the granule and appeared fibrous. In the baked bread, most of the starch was gelatinized into fibrous strands interwoven with thin, protein strands (Fig. 4).

Flour lipid droplets were scattered evenly throughout the protein strands of optimally mixed doughs but not in undermixed or overmixed doughs. We were able to differentiate between wheat flour lipids and shortening, but only the flour lipids were consistently associated with the protein. The shortening was not consistently associated with either protein or starch. Perhaps our fixation and dehydration technique extracted some lipids and disrupted the association, or perhaps the interaction is of a type not visible by electron microscopy. Finally, it is possible that our findings reflect the fact that although both flour lipids and shortening contribute to bread quality, they do so by different mechanisms.

Lipids in breadmaking

I have described several aspects of lipid-protein interaction and suggested that they are related to the contribution of lipids to breadmakins. We have many pieces that at times do not seem to fit well into the large jigsaw puzzle of the interaction among the diverse wheat flour components. The picture I visualize is that the structure or skeleton of the dough is formed by gluten proteins. The glutenins are critical to proper development of that skeleton as they govern dough mixing properties; the gliadins are essential to gas retention of the properly developed structure. The structure formed by gluten proteins can be reinforced by their interaction with lipids. The starch granules act in dough as a relatively inactive component. They are not, however, a mere diluent. Up to the stage of oven proof, strength and stability of the structure depend on the interaction of lipids and proteins. The semi-dormant starch granules become activated at the baking stage. At the critical stage of oven spring in breadmaking, major lipid interaction with starch takes place. The extent of that interaction is significant both in imparting a desirable structure to the final bread as well as contributing to overall consumer acceptance, including freshness retention.

Admittedly, our understanding of all those interactions is incomplete. To improve our understanding of those interaction products is both a challenge and a promise: a challenge because they are complex and undergo major changes in the germinating or maturing seed and in the processed cereal food; a promise, because an understanding of those interactions should provide us with a powerful tool to produce functionally improved, and nutritionally enriched cereal foods.

Practical implications in protein-enriched bread

Experiments with glycolipids synthesized in the author's laboratory (Wehrli and Pomeranz, 1969) indicated that a certain hydrophilic-hydrophobic balance is required for best effects in breadmaking (Pomeranz and Wehrli, 1969). The

significance of those findings with synthetic compounds was three-fold: they confirmed the role of natural flour glycolipids in breadmaking; they opened the possibility of synthesizing glycolipids superior to those found in flour; and they provided the basis for the production of protein-enriched bread through the addition of small amounts of glycolipids (POMERANZ *et al.*, 1969).

Wheat flour contains a fairly high concentration of proteins, but the proteins are not well balanced in amino acids and are particularly low in lysine. The quality of protein poses practically no problem if bread is part of a diet rich in protein foods of high biological value, such as milk, eggs, meat, fish, and legumes. In countries where high-protein foods are not generally available, however, wheat flour enriched with plant and animal proteins, including defatted oilseed flours, edible yeasts, and fish flour, might be used in low-priced breads and other baked products to improve nutrition.

Without the addition of extra glycolipids, even relatively low levels (about 5 p. 100) of lysine-rich protein supplements (i.e., soy flour) decrease loaf volume, impair crumb texture, and reduce shelf life. If small amounts of glycolipids are added, however, at least 10 p. 100 soy flour, containing about 50 p. 100 protein, can be included in the formula without impairing consumer acceptance of the bread. Soy protein contains about 6 p. 100 lysine (compared to only 2 p. 100 in wheat flour) ,so the addition of 10 p. 100 soy flour easily doubles the amount of nutritionally limiting lysine that would be supplied by flour. The use of glycolipids in the production of acceptable, nutritionally improved bread is particularly promising because little or no changes would be required in breadmaking processes, formulations, schedules, or equipment. The processes for producing protein-enriched bread and for synthesizing glycolipids have been granted public patents (POMERANZ and FINNEY, 1972; POMERANZ and WEHRLI, 1973); and the patents are assigned to the U.S. Department of Agriculture, which is authorized to grant royalty-free licences for their use.

Recently, the functional (breadmaking) properties of commercially available sucrose esters were improved (CHUNG *et al.*, 1976). As much as 4 p. 100 sucrose tallowate previously was required to restore breadmaking quality of high-protein breads (POMERANZ *et al.*, 1969); but now, acceptable high-protein breads can be made with as little as 0.5 p. 100 sucrose esters. The effect on loaf volume of as little as 0.25 to 0.50 p. 100 of the improved sucrose esters, added to wheat flour alone, is equivalent to the effect of 3 p. 100 shortening. On the other hand, addition of unimproved sucrose tallowate, even to wheat flour alone, is deleterious to loaf volume and crumb grain. Effectiveness of sucrose esters is proportional to their monoester contents (CHUNG *et al.*, 1976).

An " ideal " nutritionally improved high-protein bread should be low priced; require no special formulations, schedules, and equipment to produce; and be acceptable to the consumer with regard to overall eating qualities, including freshness retention. Although it is possible to produce today a high-protein breat that is superior to the one that was produced about 10 yr ago, it has not been possible to meet satisfactorily all the above requirements for our ideal bread. A recent study from our laboratories (POMERANZ *et al.*, 1977) showed that the use of flours from germinated soybeans (in the presence of small amounts of surfactants such as sucrose esters or sodium stearoyl lactylate) overcome some of the difficulties. Bread baked from 90 g wheat flour and 10 g of flour from chemically treated or germinated soybeans, in the presence of 0.50 g sucrose palmitate or 0.50 g sodium stearoyl lactylate plus 3 g vegetable shortening, was consumer-acceptable with regard to loaf volume, crumb grain, crumb color, freshness retention, taste, and

flavor. Wheat flour enriched with flour from germinated soybeans could well be the solution to the problems associated with producing low-priced, nutritionally improved, protein-enriched bread.

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Résumé

Interaction entre des lipides et les protéines du gluten

Des lipides libres de la farine de blé interagissent avec des protéines du gluten pendant le pétrissage ou la lixiviation du gluten. Le rôle de cette interaction par rapport au potentiel et aux résultats de panification ont été étudiés et les résultats ont été utilisés pour proposer des modèles.

Les lipides pourraient intervenir dans les interactions protéines-protéines et influencer des propriétés physico-chimiques des constituants qui interagissent.

Alors que les interactions lipides-protéines régissent les propriétés de la pâte, la complexation des lipides avec l'amidon a un rôle significatif dans le processus de la panification. Un aperçu sur quelques unes des modifications qui ont lieu peut être obtenu grâce à la microscopie. Les connaissances obtenues à l'aide des études sur l'interaction lipides-gluten sont utilisées pour expliquer des modifications pendant la panification. Les conséquences pratiques de ces interactions dans la production de pain enrichi en protéines sont décrites.

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The effect of heat on gluten-physico-chemical properties and baking quality

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Summary

Wheat gluten has been heated at temperatures up to 91 °C, and experiments conducted to examine the mechanism for the resultant loss of baking functionality.

Extraction of the wheat protein by a dissociating buffer containing cetyltrimethylammonium bromide, urea, sodium citrate at pH 5.5, decreased considerably as temperature of heating the gluten increased. Examination of the extracted protein by gel filtration on Sepharose CL-4B indicated that the glutenins were more susceptible to heat (i. e. rendered less extractable) than gliadins. Starch gel electrophoresis and SDS-polyacrylamide gel electrophoresis of aqueous alcohol extracts (50 p. 100 propan- 1 -01) of heated gluten showed that the only protein species unaffected by the heat were ω-gliadins, which do not contain cyst(e)ine. α, β, γ-gliadins and low molecular weight, alcohol-soluble glutenins were all rendered less extractable, particularly at the higher temperatures. Inclusion of the reducing agent dithioerythritol during alcohol extraction, however, substantially restored extractability of these components.

The results suggest that an important effect of heat is to cause conformational changes in glutenins such that new intermolecular disulphide bonds can be formed which lead to increased molecular size of the glutenin aggregates, decreased extractability and baking performance. Similar changes occur in the cyst(e)ine — containing gliadins, at the higher temperatures. The new disulphide bonds stabilise the proteins in the denatured state, on cooling, rendering the process irreversible.

Key words : *gluten — breadmaking quality — heat treatment.*

Introduction

It is well established that proteins are denatured by heating and wheat proteins are no exception to this. Of particular importance to us is the effect of heat on the proteins that constitute the viscoelastic complex, gluten. It is this component of wheat flour which is most reponsible for bread quality, that is, bread of good volume and fine, even texture. Satisfactory bread cannot be produced if the gluten protein is present at only low levels. For this reason, in the

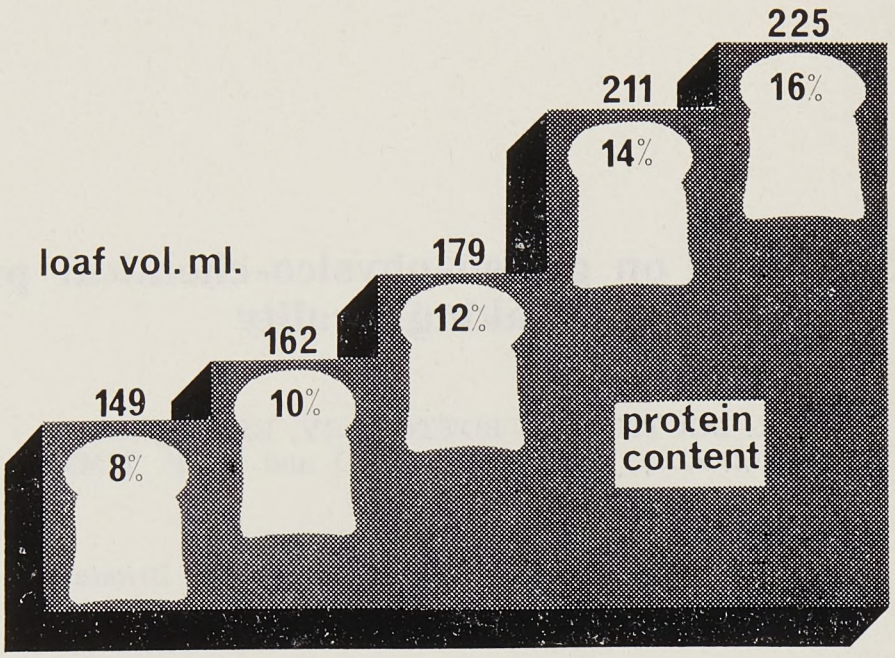

FIG. 1. — *Loaf volume increases as protein level rises in a flour reconstituted from gluten, starch and water soluble material.*

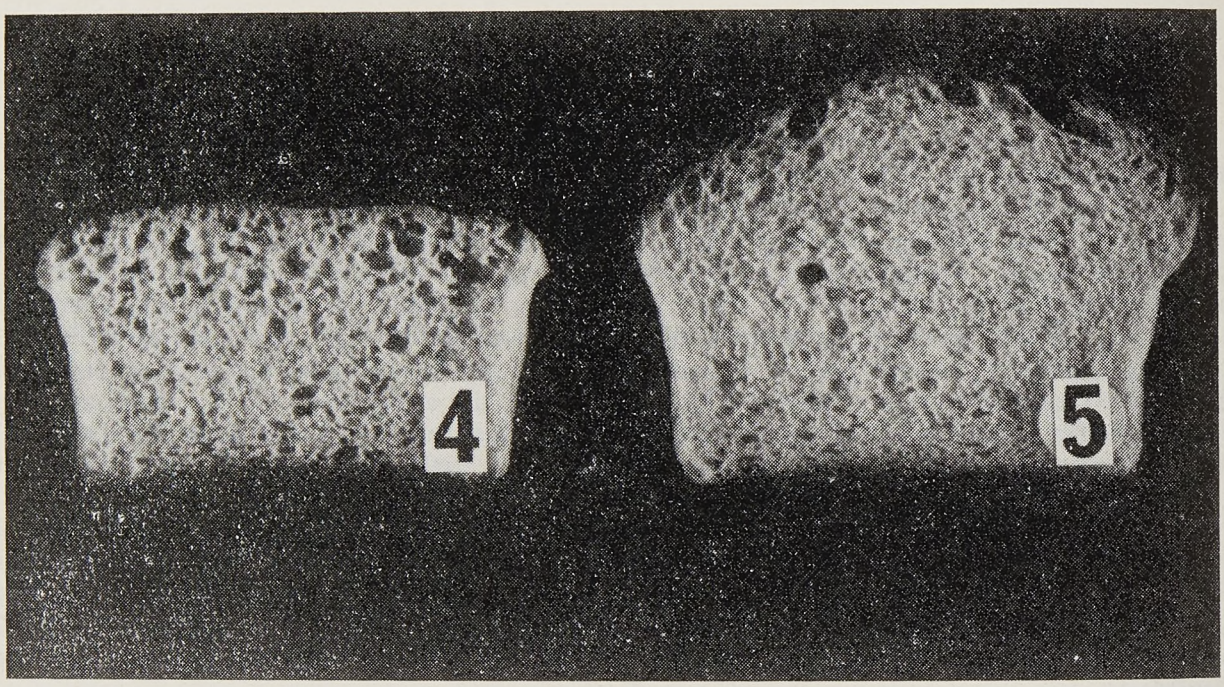

FIG. 2. — *Comparison of the baking performance of, left, Maris Huntsman, and right Canadian Western Red Spring Wheat at 10 p. 100 flour protein content.*

UK, we normally include wheat from Canada /U.S.A. in our grists — it has higher protein content than our normal home-grown wheat. Figure 1 illustrates the effect on loaf volume of increasing protein levels, obtained by baking flours which were reconstituted from starch, flour water soluble material and increasing quantities of gluten.

Low quality bread can also result when the gluten has poor functionality and it is typified by the performance of the high yielding European wheat, Maris Huntsman. This is compared in Figure 2 with bread baked from CWRS flour. These again are from reconstituted flours in order to adjust the protein contents to the same level (10 p. 100).

To the breadmaker, it is thus essential that his flour contains both sufficient

FIG. 3. — *Comparison of the baking performance of flour from, left, undenatured and right, heat denatured wheat.*

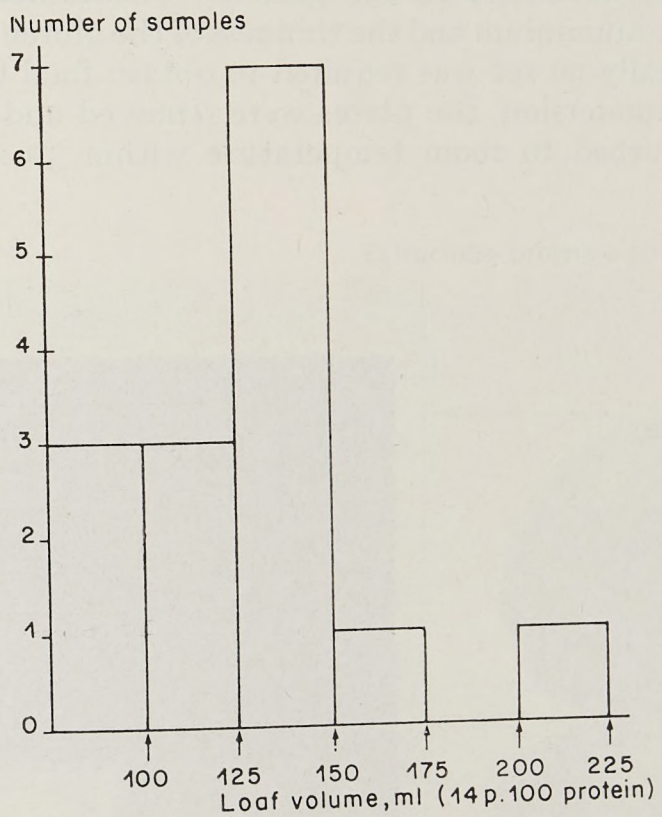

FIG. 4. — *Baking performance of commercial glutens.*

gluten protein and protein with the right quality or functionality. This protein must be unaffected by heat.

Perhaps I might mention two specific occasions when heat might affect quality. Firstly, at wet harvests, wheat grain has to be dried. Should recommended temperatures be exceeded, considerably lowered baking performance results, because of denaturation of the protein (Fig. 3).

Secondly, throughout the world, there are a number of commercial plants

that separate wheat gluten from starch. Although varying somewhat in detail, all are wet processes and the gluten has to be dried. We have examined the baking performance of the gluten from several of these plants, by making up into composite flours using standard starch and water soluble material. It is clear (Fig. 4) that although all were termed " vital wheat gluten ", the functionality as measured in this fermentation baking procedure varied considerably, implying that some had been affected by heat during drying.

The results we are going to present here are based on our studies aimed at investigating the physico-chemical changes that occur in gluten when heated.

Method

To carry out the heating we constructed parallel aluminium disc plates which could be bolted together round the outer edge. An O-ring acted as a seal. These plates are seen in Figure 5. Wet gluten (60 p. 100 moisture) was placed between the plates which were sealed 3 mm apart. For heating, to avoid moisture loss, the plates were immersed in a water bath maintained at the required temperature. Temperature of the gluten was measured by a small thermocouple probe, sealed into the plates and imbedded in the gluten. The combination of rapid heat transfer through the aluminium and the thinness of the gluten ensured rapid heat-up of the gluten, typically 90 sec was required to obtain final temperature. After a total of 5 minutes immersion, the plates were removed and plunged into ice-cold water. Gluten returned to room temperature within 30 sec. It was removed and freeze dried.

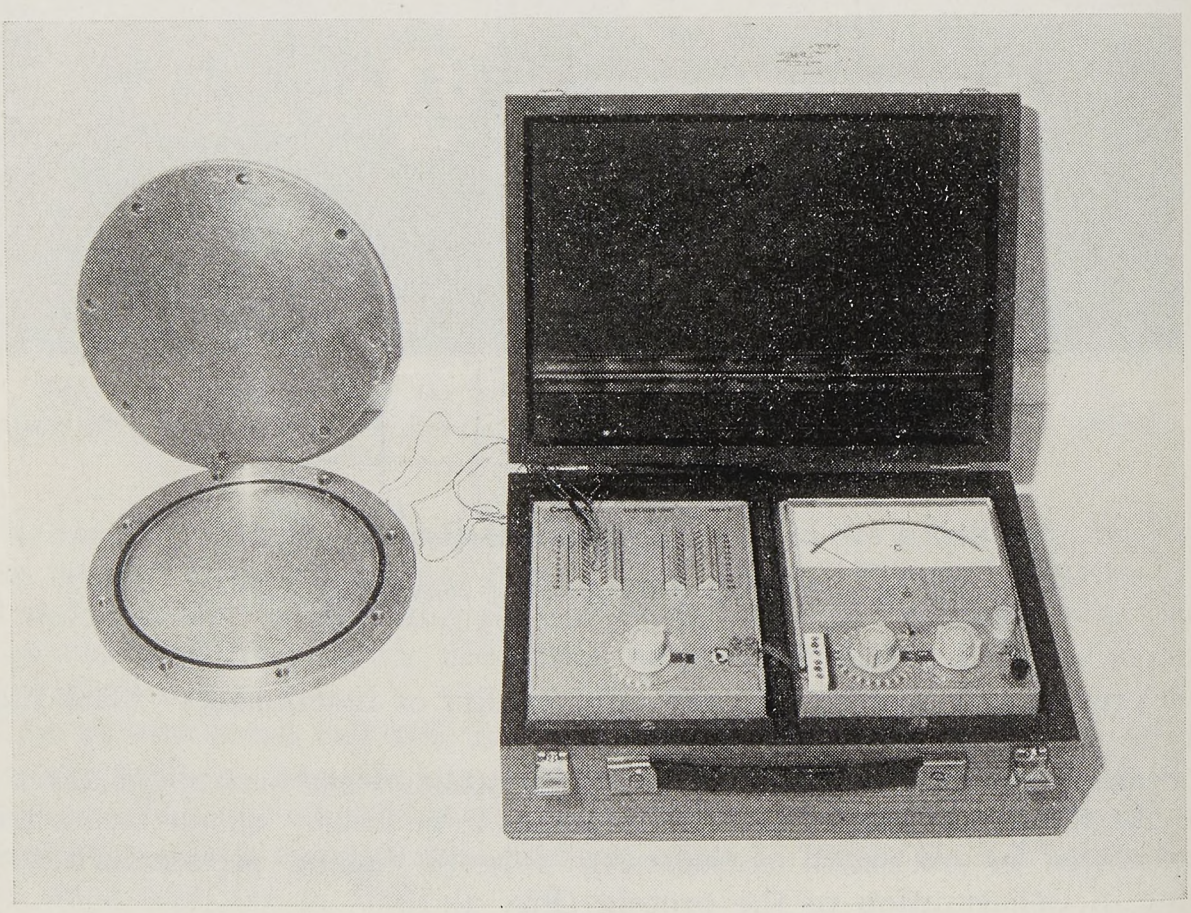

FIG. 5. — *Equipment for holding gluten during heat treatment.*

Results and discussion

Baking tests on composite flours, produced by blending the gluten with starch and flour water soluble material to 14 p. 100 protein level, showed the expected decrease in loaf volume as temperature of heating increased, particularly in the range 60-80 °C (Fig. 6).

Test involving protein extractability have been employed extensively by a number of cereal scientists to examine protein quality.

In general, on examination of commercial gluten, it has been found that quality (vitality) is positively correlated with the level of extractable protein. However the converse applies when the extractability of protein from unfractionated flour is examined. In the case of flour, the extractability of the protein decreases (i.e. the level of " residue " protein increases) as baking quality increases.

We have used solvents containing hydrogen bond breakers in order to study the changes that are brought about in gluten by heat.

In particular we have used an aqueous solution containing urea (4.5 M), cetyltrimethylammonium bromide (40 mM), and sodium citrate (50 mM) at pH5.5-CUC for short. Freeze dried gluten (125 mg) was extracted with the CUC (25 ml) by constant shaking for 18h. After centrifugation (27 000 g, 1 h, 20 °C) the super-

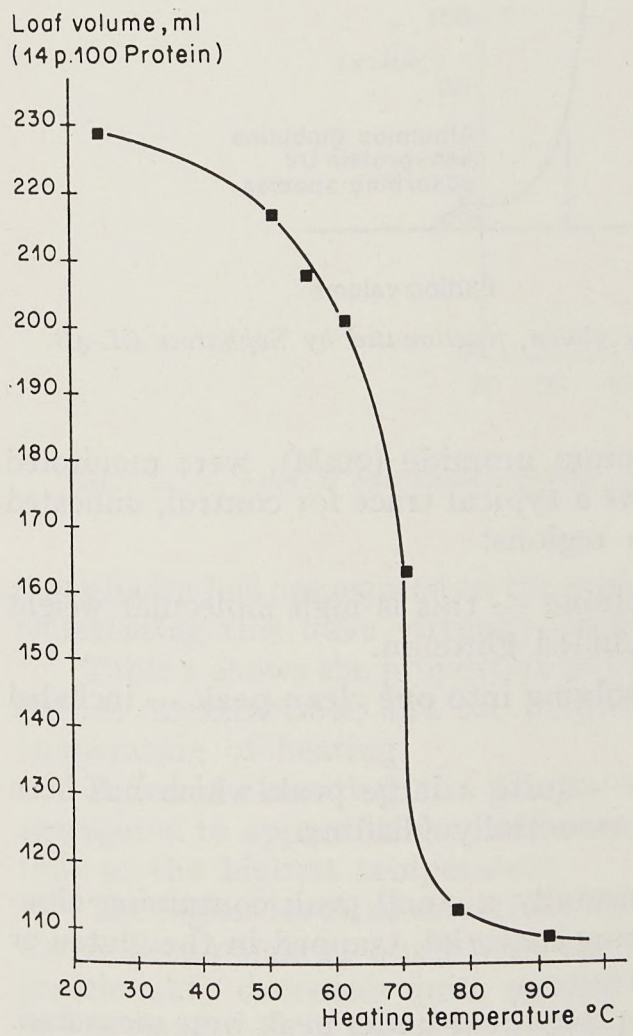

FIG. 6. — *Loss of baking quality of gluten subjected to heating.*

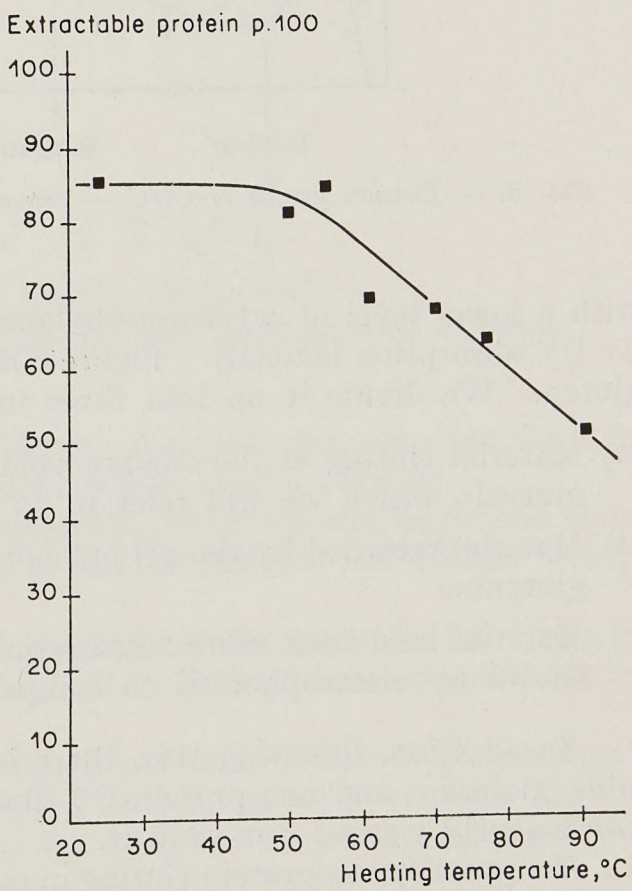

FIG. 7. — *Loss of extractable protein in gluten subjected to heating.*

natant was decanted through Whatman No. 4 paper, and retained for further studies. The proportion of original gluten protein remaining undissolved in the pellet was calculated from amino acid analysis, and hence by difference the proportion of protein dissolving in the solvent. Figure 7 shows how this dissolved protein changed with temperature of heating. A steady decrease commencing at 60 °C is seen, corresponding, you will recall, with the point at which functionality in terms of baking quality began to decrease.

We were interested in determining the change in molecular weight distribution of the gluten protein following heating. It was not possible to study further the insolubilized protein but the soluble material — the supernatant previously described — was subjected to gel filtration chromatography on Sepharose CL-4B. The proteins, eluted from the column using the CUC system but

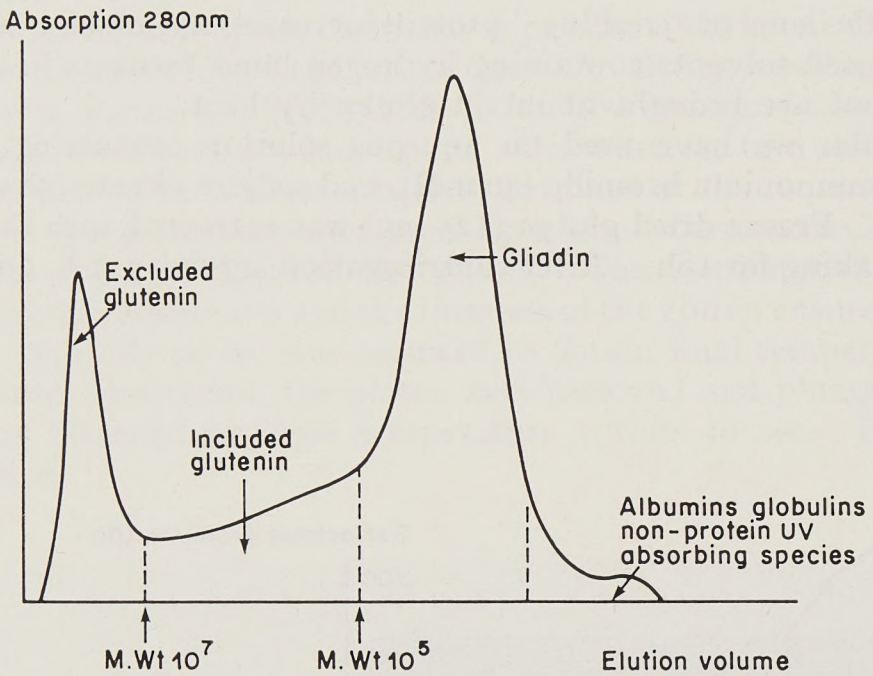

FIG. 8. — *Elution profile of CUC — extract of gluten, fractionated by Sepharose CL-4B.*

with a lower level of cetyltrimethylammonium bromide (2mM), were monitored by UV absorption (280nM). Figure 8 shows a typical trace for control, unheated gluten. We divide it up into three main regions:

(*a*) Material eluting at the column void volume — this is high molecular weight glutenin which we will refer to as excluded glutenin.

(*b*) Material retarded by the gel but not resolving into one clean peak — included glutenin.

(*c*) Material held back more substantially — quite a large peak which has been shown by electrophoresis to comprise essentially gliadins.

In addition, following this, there is normally a small peak containing albumins, globulins and non-protein-UV absorbing material, trapped in the gluten or insufficiently washed out from it.

To quantify the protein eluting in each peak, areas under peak were measured. At each temperature the size of the peak expressed as a percentage of that of the control from unheated gluten, was determined. The results are shown graphi-

cally in Figure 9. We have combined the values for excluded and included glutenin to give " Total Soluble Glutenin ". Gliadin is shown separately. Clearly, the loss of the glutenin on heating is significantly greater than that of gliadin. Very little at all of the soluble glutenin remained in the sample which had been heated to 91 °C. Still a significant proportion of the gliadin, approximately 75 p. 100, remained. This is in accordance with the general observations that large protein molecules are more heat sensitive than smaller ones and has been seen in a wide range of non-gluten proteins. The gel elution profiles show no evidence for a general increase in molecular size of the gluten proteins on heating, although this is without consideration of the insoluble gluten. It does not appear

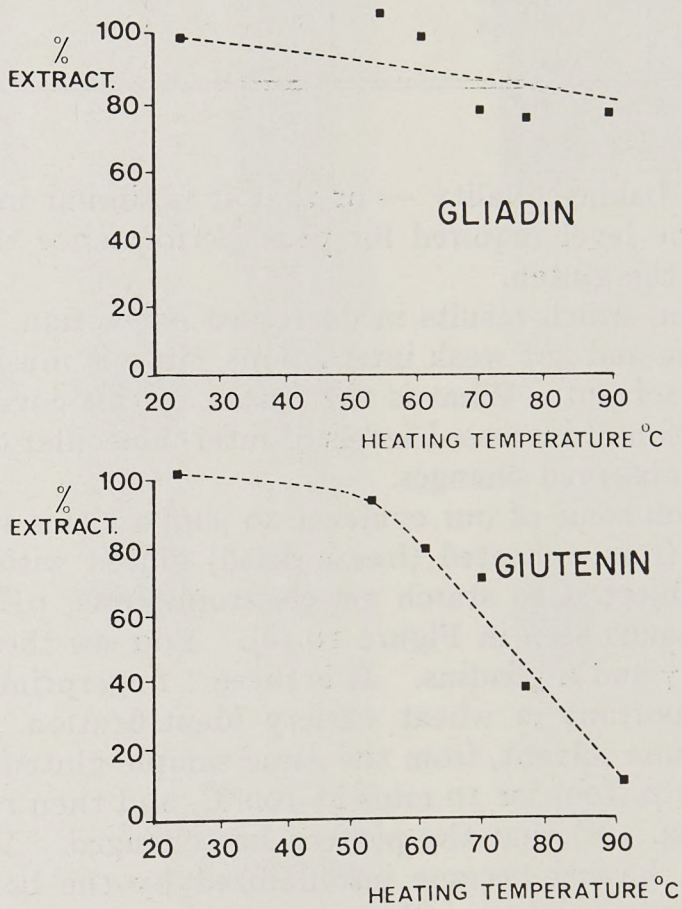

FIG. 9. — *Loss of extractable gliadin and glutenin from gluten subjected to heating.*

that gliadin has aggregated to the molecular size of soluble glutenin since the areas representing this have virtually disappeared whilst gliadin remains.

Table 1 shows the proportion of protein in the gluten existing as residue material i.e. unextractible, and the proportion of soluble glutenin and gliadin at each temperature of heating.

It seems clear that the gliadin, and particularly the glutenin, have become aggregated to appear in the residue fraction. Very little glutenin remains extractible at the highest temperature.

The higher levels of unextractible protein found in heat treated glutens do not confer better baking quality on the samples, in fact, as we have seen already, functionality decreases quite markedly as the temperature of heating rises from 60 ° to 80 °C. It is therefore apparent that either this unextractible protein differs in character from residue protein found naturally in wheat flours — ORTH and BUSHUK reported that a strong positive correlation exists between level of

TABLE I

The effect of heat on gluten fractions

Temperature °C	Residue (p. 100)	Soluble glutenin (p. 100)	Gliadin (p. 100)
24	15	28	57
55	15	25	60
61	30	20	50
70	32	21	47
77	36	13	51
91	48	3	49

residue protein and baking quality — or that it is similar in character, but present in excess of the level required for peak performance thus resulting in the tough character of the gluten.

The aggregation, which results in decreased extraction, would appear to be via covalent bonding and not weak interactions, since it must be remembered we used a dissociating solvent. What is the nature of this covalent bonding? We propose that formation of increased levels of inter-molecular disulphide bonds are responsible for the observed changes.

Let me show you some of our evidence to support this view. If we extract the soluble protein from unheated (freeze dried) gluten with 50 p. 100 aqueous propan-1-ol and subject it to starch gel electrophoresis, pH 3.2, we obtain the typical pattern of bands seen in Figure 10 (*b*). You see the complex pattern of proteins — the α, β, γ and ω-gliadins. It is these " fingerprints " that are becoming increasingly important in wheat variety identification. If we extract the protein, using the same solvent, from the same sample gluten, but now heated at natural moisture (67 p. 100) for 10 mins at 100 °C, and then run this on a starch gel, you can see (Fig. 10*c*) that the pattern has changed. Very little α, β, and γ-gliadin is present, having become insolubilized by the heat treatment. The ω-gliadins however remain intact. Now the significance of this is that the ω-gliadins completely lack the sulphur containing amino acids including, of particular importance here, cyst(e)ine.

These ω-gliadins have therefore been prevented from undergoing aggregation on heating since they lack the essential element for the change: sulphydryl and /or disulphide bonds. They remain extractable by the propanol solvent and run as normal on the starch gel during electrophoresis. The α, β, and γ-gliadins that contain cyst(e)ine, undergo conformational changes followed by re-formation of their disulphide bonds, during heating, resulting in increased aggregation and considerably decreased extractability by the solvent. If extraction of the heated gluten is repeated with propanol but now containing a disulphide reducing agent, dithioerythritol (DTE) (Fig. 10*d*) the pattern resembles very closely that obtained by extracting the unheated gluten with DTE-containing propanol (Fig. 10*a*) again suggesting the effect of heat acts via sulphydryl /disulphide groups.

We made similar observations when extracts were run on polyacrylamide gels in the presence of SDS and DTE. Those low molecular weight glutenins extracted with propanol from untreated gluten were considerably reduced when

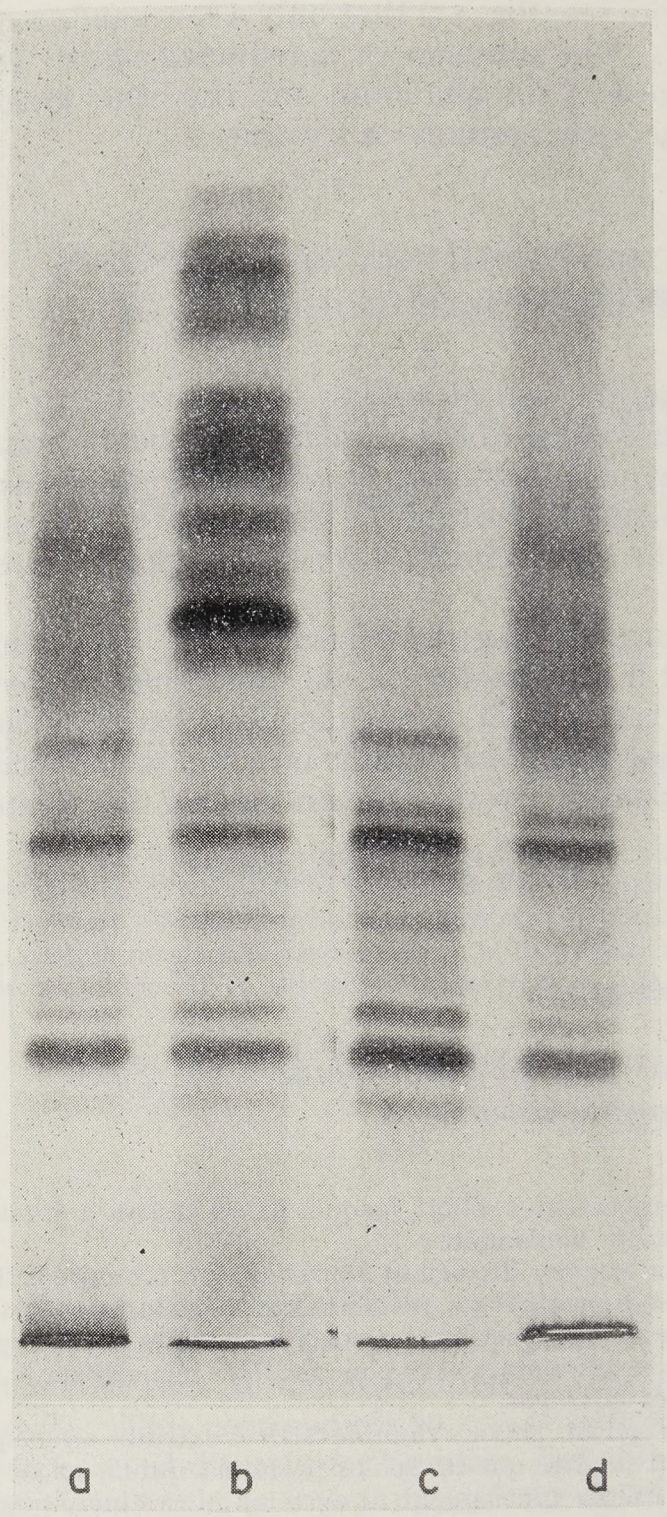

FIG. 10.

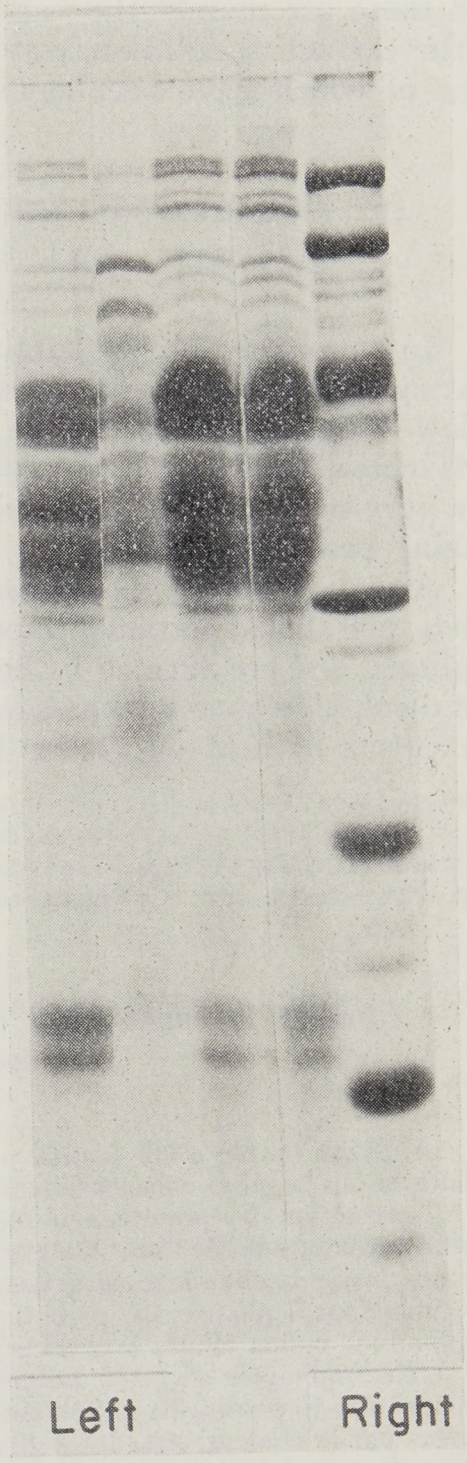

FIG. 11.

FIG. 10. — *Starch gel electrophoregrams of gliadins from gluten.*

(a) *unheated, extracted with propanol + DTE.*
(b) *unheated, extracted with propanol.*
(c) *heated to 100 °C, extracted with propanol.*
(d) *heated to 100 °C, extracted with propanol + DTE.*

FIG. 11. — *SDS-DTE-Polyacrylamide gel electropherograms of gluten proteins, left to right extracted with propanol from unheated gluten, extracted with propanol from gluten heated to 100 °C, extracted with propanol + DTE from unheated gluten, extracted with propanol + DTE from gluten heated to 100 °C; on the right molecular weight markers 94 000, 67 000, 43 000, 30 000, 20 100 and 14 400.*

extracted from heated gluten. You can see (Fig. 11) that only a few bands remain and these are mainly the ω-gliadins. The presence of a reducing agent, DTE, in the extracting solution, restores most of the glutenins, and the other gliadins, none of which have been reported to lack cysteine or cystine.

Conclusions

In summary, our proposal is that the loss of baking quality resulting from heating wheat gluten, arises through unfolding of the glutenin and gliadin proteins followed by an increase in the extent of crosslinking through disulphide bonds. This increase in extent of disulphide bonding amongst the glutenin proteins results in a very tough gluten which cannot be satisfactorily deformed during the bread-making process.

Although other types of less strong bonding, hydrogen bonds, hydrophobic bonds, are almost certainly important in the rheological and functional properties of gluten, and are affected by heat, it is the effect on the increased extent of covalent disulphide bonding which is of most functional significance and which renders the effects of heat on gluten functionality irreversible on cooling the protein.

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Résumé

Effet de la chaleur sur les propriétés physico-chimiques du gluten, et sur sa qualité boulangère

Le gluten de blé a été chauffé à des températures allant jusqu'à 91 °C et l'on a étudié le mécanisme de la perte consécutive de sa qualité boulangère.

L'extraction des protéines de blé par un tampon dissociant contenant du bromure cethyltrimethylammonium, de l'urée, du citrate de sodium à pH 5.5., diminue considérablement lorsque la température de chauffage du gluten augmente. Le fractionnement des protéines par tamisage moléculaire sur Sépharose CL-4B indique que les gluténines sont plus sensibles à la chaleur (c.à.d. sont rendues moins faciles à extraire) que les gliadines.

L'électrophorèse en gel d'amidon et en gel de polyacrylamide-SDS d'extraits alcooliques (propanol 1 à 50 p. 100) de gluten chauffé ont montré que les seules protéines qui ne soient pas affectées par la chaleur, sont les ω-gliadines, qui ne contiennent ni cystéine ni cystine. Les gliadines α, β, γ et les gluténines alcoolo-solubles (de poids moléculaire faible), deviennent toutes moins faciles à extraire, et plus particulièrement aux températures les plus élevées. L'addition d'un agent réducteur, le dithioérythritol, lors de l'extraction alcoolique, restitute toutefois à ces constituants l'essentiel de leur solubilité.

Les résultats laissent penser qu'un des effets important de la chaleur est de provoquer des changements de conformation des gluténines, tels qu'ils permettent la formation de nouveaux ponts disulfures intermoléculaires, ce qui augmente la masse moléculaire des agrégats de gluténine, et diminue leur extractibilité et la qualité en panification. Des transformations similaires se produisent dans les gliadines contenant de la cystéine ou de la cystine aux températures les plus élevées. Ces nouveaux ponts disulfures stabilisent les protéines dans leur état dénaturé, lors du refroidissement, rendant ainsi le processus irréversible.

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Functional properties of corn germ protein related to the interaction with water and with fats

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Summary

Proteins were extracted from the flour of defatted corn germ residual from the oil industry. They are scarcely soluble at neutral or slightly acid pH but dissolve well in alkaline or more acidic medium. Water sorption is extremely high and the sorption-desorption process is reversible. Emulsifying properties are also good, with a sharp minimum at pH 5.5 Fat absorbtion is quite high as compared to other food proteins.

Key words : *proteins — wheat germ — functional properties — water — lipids.*

Corn germ is potentially a copious source of protein for human uses. Moreover it is the source of oil of good quality. At present an yearly average of 9 million metric tons of non degerminated corn flour are used in Italy mostly as animal feed ([1]). This corresponds to 170 000 tons of protein, part of which might profitably be used directly for humans, and to large quantities of oil of valuable nutritional composition which become saturated fats in the animal.

Much of the proteins, oil, and tocopherols are associated with the germ. Degermination is performed by wet processing for starch production to an extent corresponding in Italy to 8 000 tons of protein per year and by dry processing for oil extraction, the equivalent of 7 000 yearly tons of protein. This practice however is like to increase because of increased request for germ oil and of economical incentives. The total amount of corn used yearly in Italy would allow to isolate germ with a protein content approaching 190 000 tons.

From the nutritional standpoint germ proteins have interesting capacities. They represent about 15 p. 100 of dry weight in defatted germ deriving from dry separation and 18 p. 100 in that deriving from wet separation (C. PERI *et al.*, 1980). The modified essential aminoacid index MEAA is 65 p. 100 as compared to the provisional essential aminoacid pattern proposed for human uses by FAO-WHO, enzymic digestion according to Satterlee (SATTERLEE *et al.*, 1979) is 76.5 p. 100,

the enzymic ultrafiltrate digest (E.U.D.) is 67 as compared to 46 for defatted sunflower, 52 for protein concentrate from favabean and 78 for that from blood([3]). Calculated PER is 2.15 and limiting aminoacids are isoleucine followed by sulfurated aminoacids (F. FIDANZA, 1779).

Breakdown of germ proteins by endogenous proteolytic enzymes is only marginal: indeed the germ contains only peptidases and protease activity is located in the endosperm and becomes evident only two days after germination (T. MOUREAUX, 1979). Separation of germ from endosperm protects therefore its proteins.

Studies for which this preliminary report is given were intended to ascertain some aspects of the behaviour with water and with fats of proteins extracted from germ residual from the oil industry so as to make clear their possible technological use and if required to improve the separation procedures of the germ. Technological studies were carried out in parallel (C. PERI *et al.*, 1980).

Materials and methods

Proteins were assayed with the microkjeldahl method (A.O.A.C. 38, 012, 10th edition) after precipitation with trichloroacetic acid, using 6.25 as conversion factor.

Solubility was determined according to Kinsella (J. C. WANG et J. E. KINSELLA, 1976) with some modifications: the suspension was stirred 2 hours at 25 °C and was then centrifuged 45 min at 7 800 × g.

Water adsorption and desorption was measured according to HUFFMAN *et al.* (1975) at 20 °C. Potassium acetate, magnesium nitrate, ammonium sulfate and dibasic sodium phosphate were used to produce specified relative humidities in the range from 20 to 95 p. 100. Measured values coincided with values reported on the Merck Index.

Emulsion properties were assayed according to Yatsumatsu (YATSUMATSU *et al.*, 1972) ad 2 ml samples containing 140 mg protein. Kinsella had already applied this method on small amounts of proteins. Samples were shaken at 710 cycles per minute with an Homogenizer drive shaker of Thomas.

Fat binding was measured as described by LIN *et al.* (1974). The germ was separated by shock treatment of the kernel (M. CINQUETTI, 1978) and further processed on the dry line: this involves wetting and heating the material at 95-100 °C, relative humidity 12-13 p. 100, followed by drying at 115 °C. These operations require together 20 min. The dry germ was laminated and then extracted with hexane; the residual solvent was removed at temperatures between 80 and 115 °C, with about 20 min. heating. The final product contained fat, 1.5 p. 100 of dry weight, and 17.8 p. 100 total nitrogen 86.7 p. 100 of which was protein nitrogen. Nitrogen solubility as measured by the nitrogen solubility index, NSI, decreased about 40 p. 100 during heat conditioning whereas practically no change was observed during the other operations mentioned.

Proteins were extracted by shaking for one hour at room temperature 30 gm germ flour, particle size 60 mesh, in 100 ml 70 p. 100 aqueous ethanol. Diluted NaOH (330 ml), final concentration 0.5 p. 100, was then added and the extraction was continued overnight. The suspension was then centrifuged 5 min at 12 000 × g: 68 p. 100 of total nitrogen of the germ was thus recovered of which 75 p. 100 was protein nitrogen. The supernatant was dialyzed for two days at 2-4 °C and then frozen-dried: protein represented 88 p. 100 of this material.

Results and discussion

Solubility

The amount of protein soluble at various pH is shown in table I. Solubility is low in neutral or slightly acidic medium and is minimal, with only 20 p. 100 protein dissolved, at pH 5.0. On the other hand it is good in alkaline medium and also at more acidic pH. Increasing the ionic strength at the pH of minimum solubility affects only slightly the amount of protein dissolved: maximal effect is at

TABLE I

Effect of pH (A) and of ionic strength (B) on solubility of corn germ proteins

Germ proteins (100 mg) were suspended: A) in 10 ml water at the desired pH, the final ionic strength being made 0.02 with NaCl or B) in 10 ml water at pH 5.0, the ionic strength being adjusted at the desired value with NaCl. The suspensions were shaken 2 hours at 25 °C the pH being controlled and the undissolved material was then centrifuged off at 7 800 × g for 45 min. Total nitrogen was determined in the supernatant.

A		B	
pH	protein solubilized (p. 100)	I	protein solubilized (p. 100)
2	85.2	0.01	21.9
3	39.9	0.05	22.9
4	27.8	0.10	24.3
5	22.1	0.40	27.8
6	50.0	0.50	28.1
7	68.2	0.80	28.2
8	84.1		
9	92.8		
10	97.5		
11	99.8		

ionic strength between 0.1 and 0.4, the same range where globulins from other sources, e.g. lupine seed, are solubilized (P. Gerletti *et al.*, 1980).

Different authors give somewhat different values for solubility classes of proteins in corn germ: albumins respectively 30 and 40 p. 100, globulins 30 and 13 p. 100, zein 5 and 2.5 p. 100, glutelins 25 and 18 p. 100 (P. A. Reiners *et al.*, 1973), (J. Landry, T. Moureaux). The specific pattern depends however on the state and on manipulations of the seed since salt soluble proteins aggregate to G-3 glutelins at the beginning of maturity and disaggregate during germination and it has been suggested that vigorous stirring and use of water as first extractant brings about in endosperm a partial insolubilization of salt-soluble proteins and of G-3 glutelins These observations give reason of the solubility characteristics found in our work. Differences with respect to data reported may be attributed to some modification

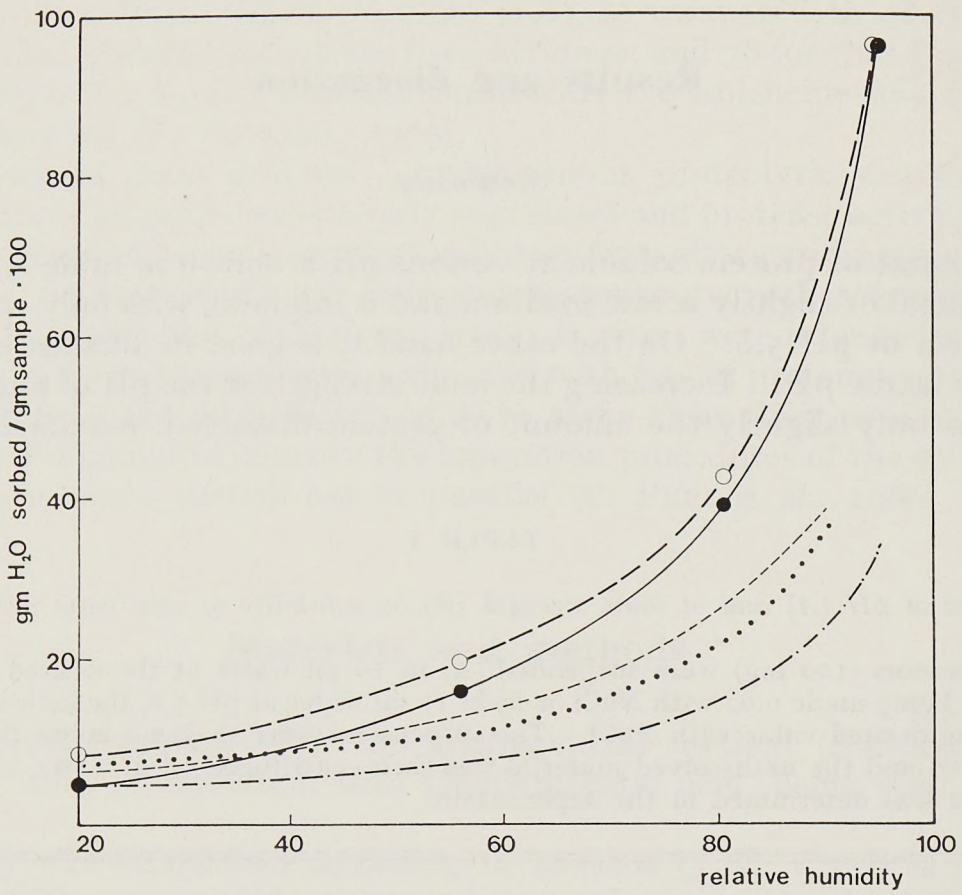

FIG. 1. — *Sorption and desorption behaviour of corn germ proteins.*

Germ proteins were dried at 50 °C for four days and then equilibrated at 20 °C in a desiccator in presence of saturated salt solutions of known relative humidity. The relative humidity was checked throughout with an hygrometer. The data on germ proteins are compared with those on other proteins, obtained in our laboratory (lupine globulins, —.—) or reported in the litterature: Promine D..., whey protein concentrate — — —, both measured at 15 °C; ref. 15.

of the proteins during germ treatments previous to protein extraction. As previously mentioned heat conditioning causes denaturation, which gives reason also for the only partial extraction of proteins from the flour.

Water adsorption

The amount of water adsorbed is very high reaching at 95 p. 100 relative humidity 1 gm per gm protein. As shown in Figure 1 the sorption capacity of corn germ proteins largely overcomes that of other food proteins. The data quoted for Promine D and whey proteins refer to 15 °C and it is known that less water is adsorbed when temperature increases (A. M. HERMANSSON).

Comparison of sorption and desorption curves indicates that hysteresis is quite small, which speaks in favour of the reversibility of the process.

Emulsifying properties

The amount of emulsion produced and the stability of emulsion are quite good in all the pH range assayed except for a minimum at pH 5.5 (Table 2). The minimum is slightly displaced towards neutrality with respect to the pH of mini-

TABLE 2

Emulsion properties of corn germ proteins

Germ proteins (140 mg) suspended in water and adjusted to the desired pH and to a final volume of 2 ml were shaken for 1 min. at 710 revolutions per min. with 2 ml soy oil, then were centrifuged for 5 min. at 1 200 × g. The height of emulsion layer, as percent of the total content of the tube, measures the emulsion activity. An equivalent measure made after keeping the tube 30 min. at 80 °C and 15 min. at 15 °C before centrifuging gives the stability of emulsion.

pH	Emulsion acitivity	Stability of emulsion
2.0	45.9	48.1
4.0	50.0	55.4
5.0	49.5	56.9
5.5	9.3	12.3
6.0	49.5	53.9
8.0	52.6	49.5
11.0	49.2	50.5

mal solubility and is quite sharp, goad emulsifying properties being displayed at pH values where solubility is reduced. This indicates that the emulsion behaviour is not directly related to the solubility properties of the proteins.

The ability of part of the proteins to disperse at the interface between oil and water phase stabilizing the emulsion suggests that these molecules dissociate and unfold and that part of peptide has hydrophobic character.

Fat absorption is compared in table 3 with litterature data for other food proteins, measured under similar conditions. The performance of proteins from corn germ is very good.

The data here reported give only preliminary information on the interaction

TABLE 3

Fat absorption of corn germ proteins

Corn germ proteins (500 mg) were shaken for 1 min. in a centrifuge tube on a Vortex vibrator with 3 ml soy oil. They were then kept at 24 °C for 30 min. After centrifuging for 25 min. at 1,610 × g the volume of free oil was measured. Fat absorbed is expressed as ml oil bound (gm sample)$^{-1}$. 100.

	Fat absorption
Corn germ proteins	549
Lupine globulins	190
Soy concentrates and isolates (10)	From 92 to 154
Sunflower concentrates and isolates (10)	From 227 to 256
Alfa alfa leaf protein preparations (7)	From 180 to 430

of corn germ proteins with water and with fats. As a first approach to functional properties of this new source of food protein, they appear promising and justify reasonable hopes for possible technological applications.

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Résumé

Les protéines du germe de maïs : propriétés fonctionnelles découlant de leur intéraction avec l'eau et les lipides

Les protéines du germe de maïs ont été extraites à partir d'un tourteau délipidé, résidu de l'industrie huilière, avec une solution aqueuse d'éthanol (70 p. 100) et de NaOH (0,5 p. 100). Peu solubles à pH neutre ou légèrement acide, ces protéines se dissolvent aisément dans un milieu basique ou fortement acide. Elles ont une très grande capacité d'absorber l'eau et le processus d'adsorption et de désorption est réversible. Leurs bonnes propriétés d'émulsionnage passent par un minimum à pH 5. Leur capacité d'adsorption des graines est plus élevée que celles des autres protéines alimentaires.

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Méthode de dosage des nitrites et des nitrates applicables aux produits carnés

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avec la collaboration technique
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Résumé

La présente étude décrit deux méthodes de dosage des nitrites et des nitrates.

Les nitrites sont dosés en utilisant la coloration de GRIESS avec l'acide sulfanilique et la N-(1-Naphthyl) éthylènediamine comme couple de réactifs et lecture à 530 nm dans une méthode manuelle, la sulfanilamide et la N-(1-Naphthyl) éthylèndiamine avec lecture à 540 nm dans une méthode automatique.

L'extrait par HCl 0,05 N des nitrates est purifié par passage sur colonne de résine IR 45 pour en éliminer une fraction qui conduisait à une réduction non quantitative des nitrates sur colonne de cadmium.

La colonne de réduction utilisée est un amalgame de cadmium et de mercure (méthode manuelle) ou de cadmium et de cuivre (méthode automatique).

Dans la méthode automatique la colonne de résine est remplacée par une dialyse en continu.

La cysteine et plus faiblement la cystine inhibent partiellement la réduction des nitrates sur la colonne de cadmium si l'on omet la colonne de résine.

La technique manuelle permet de doser le nitrite en concentration supérieure à 2,5 mg de NO_2Na par kilogramme de produit frais avec une précision de 2 p. 100. Les nitrates peuvent être dosés à partir d'une concentration de 5 mg de NO_3K par kilogramme de produit frais avec une précision au moins égale à 7 p. 100. Les précisions de 2 et 7 p. 100 ne tiennent pas compte de l'hétérogénéité de l'échantillonnage.

La précision de la méthode automatique est supérieure à 2 p. 100. L'étendue de mesure est de 1 à 10 pour chaque rapport de tube échantillon /diluant soit au total 1 à 1 500 environ. Le seuil de détection se situe à environ 1 mg /kg de produit frais pour les nitrites et les nitrates.

Mots clés : *nitrites, nitrates, viande, produits de viande.*

Introduction

Les nitrites et les nitrates sont des additifs classiques des salaisons. La forme active est le nitrite employé directement ou obtenu après réduction bactérienne du nitrate. Le dosage de ces composés est nécessaire pour toutes les études portant sur des produits de salaisons. Ils interviennent à la fois : sur la coloration, la flaveur, sur l'orientation des fermentations et comme inhibiteurs du développement des *Clostridia* (Bousset et Fournaud, 1976).

De plus, la formation éventuelle de nitrosamines à partir des nitrites soit dans les produits, soit *in vivo* chez l'homme, oblige à contrôler de façon très rigoureuse les quantités résiduelles.

Il est donc nécessaire de disposer de méthodes d'analyses fiables de ces deux composés.

Considérations sur les méthodes existantes

Pour le dosage des nitrites, les difficultés se situent au niveau de leur extraction totale car une partie est plus ou moins liée aux différents constituants des charcuteries, essentiellement aux protéines. Une fois extraits ou libérés leur dosage ne pose que très peu de problème si l'on admet de doser dans un même ensemble NO_2^- et NO.

Les techniques les plus généralement employées sont basées sur la coloration de Griess (1879) (formation d'un sel de diazonium en milieu acide puis copulation de ce sel avec une amine aromatique pour donner un dérivé coloré). Pour obtenir la coloration on emploie des réactifs variés en recherchant parmi les produits les moins dangereux possibles pour les analystes, ceux donnant un composé coloré soluble dans l'eau, stable à la lumière et dont la formation soit, dans de larges variations de concentration, proportionnelle à la quantité de nitrite.

Dans le cas des nitrates, l'extraction est réalisée de façon apparemment quantitative par les techniques employées mais leur dosage n'est pas satisfaisant dans de nombreux produits et en particulier les viandes et les charcuteries.

De très nombreuses voies ont été explorées pour le dosage direct des nitrates à l'état de NO_3^- mais peu se sont révélées exploitables.

De par la nature du produit analysé on doit toujours doser les nitrates en présence de composés de nature organique de faible poids moléculaire que ni la chaleur ni les différents défécants ne parviennent à éliminer totalement.

De ce fait, des méthodes comme celles au Xylenol sont très difficiles à mettre en œuvre. Barnes (1950) réalise la distillation du produit coloré pour éliminer la matière organique mais dans ce cas, une partie des nitrates reste bloquée (Cserhati 1970/1971) et de plus il faut avant de développer la coloration éliminer les chlorures et les nitrites (Harley et Asai, 1963).

L'emploi de la brucine a été proposé pour le dosage direct des nitrates. Mais le produit est très dangereux et il présente les mêmes types d'inconvénients, interférence de la matière organique (Baker 1967, Fisher *et al.* 1958), des nitrites (Robinson 1958).

Les techniques pondérales comme celle de Busch (1905) employant le nitron ne sont pas assez sensibles même avec la modification de Rae et Hall (1963) qui

dose le nitrate précipité après réduction en ammoniac. Les nitrites interférent dans cette méthode et donc elle ne peut être appliquée aux charcuteries.

Un réactif comme le I Aminopyrène, (Sawicki, 1963) est très réactif mais le fer est le principal interférent. La méthode à l'antipyrine a également été développée (Bermejo et Zunzunegui, 1972) avec plus ou moins de succès.

Les essais d'emploi du sulfate ferreux datent de 1835, (Desbassins de Richemont). Ils sont périodiquement repris (Bailhache, 1889; Morris, 1958) sans qu'une méthode définitive en résulte, les nitrites interfèrent dans ces méthodes.

Certaines méthodes résultent de la généralisation de techniques éprouvées comme par exemple celle de Van Slyke et Lomonte (1969) par gazométrie mais qui n'est pas suffisamment sensible, ou celle de Conway (1956) délicate à mettre en œuvre.

Plus prometteur était depuis 1968-1969 l'emploi d'électrodes « spécifiques » malheureusement les résultats sont décevants (Paul, 1968; Bunton, 1969), (Manahan, 1969; Mergey, 1978) et l'on constate de plus en plus que de nombreuses substances interfèrent, ce qui conduit à des techniques très compliquées de préparation d'échantillon.

De très nombreuses autres méthodes ont été également proposées mais elles ne sont pas applicables dans le cas des produits carnés : méthode de Cawse (1967) (absorption dans UV), de Bradstreet (1965) (transformation en amoniaque), de Bloomfield *et al.* (1965) (interférence des nitrates dans le dosage du Rhenium) automatisé par Holz et Kremer (1970).

Les méthodes directes de dosage des nitrates ne pouvant pas être appliquées de nombreux auteurs ont tourné la difficulté en dosant les nitrites obtenus par réduction des nitrates.

La réduction peut se faire par voie enzymatique d'origine végétale (Bergersen, 1961; Lowe et Hamilton, 1967), ou bactérienne (Mohan, 1975). La préparation de l'enzyme est laborieuse et l'on a plus généralement recours à la réduction par voie chimique.

Dans les méthodes automatiques on emploie très couramment l'hydrazine mais si l'on traite des produits différents la mise au point est assez délicate pour obtenir une réduction quantitative.

La technique la plus généralement utilisée est celle utilisant le cadmium proposée par Grau et Mirna (1957) et améliorée par Follet et Ratcliff (1963). La commercialisation du cadmium pour réduction permet maintenant de se dispenser de sa préparation qui était assez délicate pour obtenir des résultats reproductibles. Rebelein (1968) avait proposé simplement d'agiter le produit avec du cadmium puis de filtrer, mais Mirna (1969) a montré que dans le cas des produits carnés la technique n'est pas satisfaisante, soit par réduction insuffisante, soit au contraire par récupération anormale de nitrate. Les résultats de Volff *et al.* en 1974 font apparaître également que la méthode par agitation du cadmium ajouté dans l'extrait est délicate à mettre en œuvre. On est donc conduit à utiliser des colonnes mais le cadmium pur ne fournit pas une réduction parfaitement complète, Graszhoff (1964) a proposé l'emploi d'un amalgame de cadmium et de mercure qui fournit des résultats beaucoup plus reproductibles. Il en est de même dans les produits laitiers avec l'emploi de la technique de Brathen et Svenden (1973) qui associe le cadmium au cuivre préconisé par Wood *et al.* (1967). L'emploi du cuivre est moins dangereux que celui du mercure.

Les études menées dans notre laboratoire, qui impliquent de nombreuses détermination de nitrites et de nitrates dans les produits de charcuterie et de salaison, nous ont amené, à l'issue des difficultés rencontrées, à apporter diffé-

rentes modifications aux techniques existantes. Ce travail nous conduit à la proposition de techniques nouvelles, une manuelle et une automatique.

Les techniques proposées pour le dosage des nitrites sont celles classiquement utilisées. Les modifications portent sur les couples de réactifs de coloration. Elles ne dosent que les nitrites « libres » ou « libérés ».

Les améliorations apportées au dosage des nitrates concernent dans les deux techniques l'emploi d'amalgame de cadmium pour rendre la réduction reproductible et quantitative et dans le cas de la technique manuelle le passage sur résine des extraits à analyser pour en éliminer la fraction qui conduit à une réduction non quantitative des nitrates lors du passage du cadmium.

Ces techniques sont exposées ci-après. A la suite de chacune d'elle on a discuté certaines étapes des dosages.

I. — Proposition d'une méthode manuelle de dosage des nitrites et des nitrates

1.1. *Matériel et méthode*

1.11. *Échantillons*

Les analyses sont effectuées sur des échantillons frais ou congelés. Les échantillons sont hachés finement dans un « microcutter ».

Les valeurs trouvées pour les échantillons congelés ne sont pas forcément les valeurs absolues des échantillons initiaux.

1.12. *Mode opératoire*

1.121. *Solubilisation des nitrites*

La préparation des échantillons est réalisée selon la méthode proposée par l'AFNOR.

1.122. *Solubilisation des nitrates*

Une dilution de viande au 1/10 (P/V) dans de l'acide chlorydrique 0,05 N est portée au bain-marie à 70 °C pendant 20 mn pour précipiter les protéines, puis filtrée.

1.123. *Purification des extraits de nitrate*

— *traitement de la résine et préparation des colonnes*

La technique est essentiellement celle décrite par Bousset-Fatianoff et Gouet (1971) dans laquelle on remplace la résine IR4B dont la fabrication est très limitée par la résine IR 45 de caractéristiques voisines.

Réaliser quatre traitements successifs avec H_2O, HCl N, Na OH 0,5 N, HCl N et terminer le rinçage par HCl 0,05 N.

Les colonnes ont un diamètre intérieur de 1,2 cm et une hauteur de 10 cm et elles sont pourvues d'un siphon et d'un réservoir de 300 ml (fig. 1). Il est intéressant que le siphon soit muni de 2 pointes amovibles, une réglée pour un débit d'environ 1,5 ml/mn, l'autre d'environ 5 ml/mn. Les colonnes sont remplies sur 6 cm de hauteur avec la résine sous forme Cl^- en suspension dans HCl 0,05 N. Rincer successivement avec 50 ml de HCl 0,05 N à 5 ml/mn, 50 ml NH_4OHN à 1,5 ml/mn, 50 ml H_2O à 5 ml/mn, 50 ml HCl N à 1,5 ml/mn, 50 ml HCl 0,05 N à 5 ml/mn. Répéter l'opération 3 fois. Si la colonne gonfle après le passage de HCl N remettre toute la résine en suspension avec une tige de plastique dans la colonne. Les colonnes prêtes peuvent être conservées sous HCl 0,05 N.

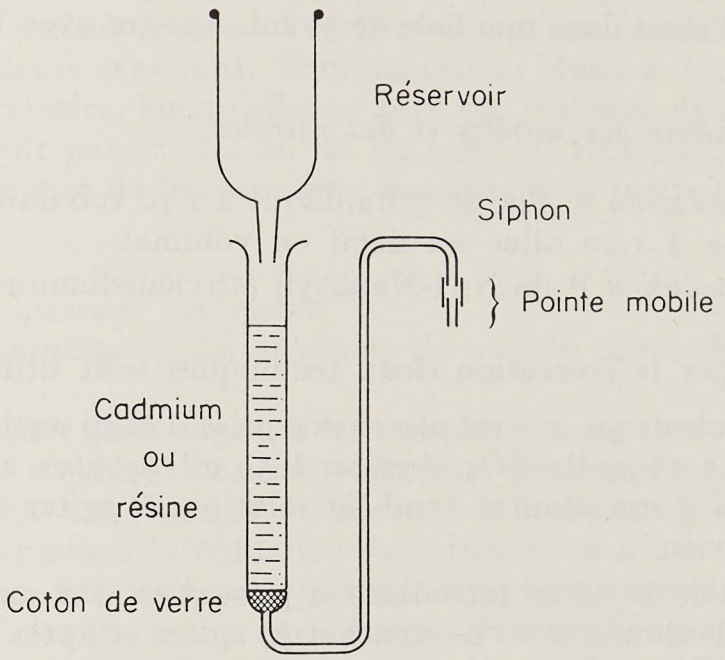

FIG. 1. — *Schéma des colonnes de cadmium ou de résine.*

— *Traitement des extraits*

Une colonne peut retenir 15 500 µg de NO_3^-.

Faire donc passer de 20 à 300 ml d'extrait à 1,5 ml/mn. Rincer la colonne par 300 ml HCl/0,05 N au débit de 1,5 ml/mn pendant 10 mn puis 5 ml/mn. Jeter le liquide de rinçage. Placer une fiole de 100 ml sous le siphon et éluer les nitrates par 50 ml de NH_4OH N à 1 ml/mn suivis de 50 ml d'eau à 5 ml/mn. Ajuster éventuellement la fiole à 100 ml.

Entre deux passages d'extrait régénérer la colonne par 100 ml de HCl N à 1,5 ml/mn suivis de 50 ml HCl 0,05 N à 5 ml/mn.

1.124. *Réduction des nitrates*

— *Traitement du cadmium et préparation des colonnes de réduction*

Traiter 50 ml de cadmium pour réduction (MERCK réf. 2001) successivement par 200 ml H_2O, 200 ml HCl 0,1 N et 200 ml H_2O puis agiter environ 50 ml de cadmium avec 100 ml d'une solution de $HgCl_2$ à 1 p. 100. Rincer par H_2O, HCl 0,1 N et H_2O en maintenant en permanence sous les liquides.

Remplir des colonnes identiques à celles utilisées pour la résine avec le cad-

mium ainsi préparé; le remplissage s'effectuant sous eau jusqu'à une hauteur de 9 cm. Placer un réservoir de 50 ml et régler la pointe des siphons pour des débits de 3 à 5 ml/mn.

Avant et après chaque échantillon faire passer sur la colonne 2 × 20 ml H_2O, 20 ml HCl 0,1 N, 2 × 20 ml H_2O, 20 ml de tampon 9,7 (0,3 N en NH_3; 0,2 N en HCl).

Les colonnes de réduction se conservent bien sous tampon 9,7 en les bouchant pour éviter l'évaporation.

— *Réduction des échantillons*

Passer sur la colonne 20 ml de solution à réduire, rincer par 4 ou 5 fois 4 ml d'eau et recueillir l'éluat dans une fiole de 50 ml. Ajuster avec H_2O jusqu'à 50 ml.

1.125. *Colorimétrie des nitrites et des nitrates*

Préparer une *solution* A d'acide sulfanilique à 1 p. 100 dans HCl environ 6 N (acide chlorydrique d 1,19 dilué au demi en volume).

Préparer une *solution* B de N-(1-Naphtyl) éthylènediamine HCl à 0,1 p. 100 dans H_2O.

Pour développer la coloration deux techniques sont utilisables :

— soit dans une fiole de 50 + 5 ml placer une prise d'essai ne dépassant pas 20 ml et contenant 3 à 25 µg de NO_2^-. Ajuster à 50 ml, ajouter 2 ml de solution A et agiter; après 3 mn ajouter 1 ml de solution B, agiter et lire la coloration après 15 à 30 mn;

— soit dans une fiole de 50 ml introduire la prise d'essai et amener à un volume d'environ 40 ml, ajouter 2 ml de solution A, agiter et après 3 mn ajouter 1 ml de solution B, ajuster à 50 ml, agiter et lire après 15 à 30 mn.

Dans les deux cas introduire un standard et un blanc dans chaque série de lecture.

1.2. *Observations*

1.21. *Réactifs de coloration des nitrites*

L'emploi de l'acide sulfanilique et de la naphtylamine de Griess 1879 ne nous donnait pas entière satisfaction : la coloration était peu stable.

La sulfanilamide comme agent de formation du dérivé diazo et le couplage avec la N-(1 Naphtyl) éthylènediamine HCl donnent une coloration stable à la lumière pendant plusieurs heures. Mais les résultats n'étaient pas reproductibles. Nous pensons que ceci est dû à la formation d'un composé entre l'extrait de viande et la sulfanilamide. Ce composé absorbe très fortement à 440 nm. Cette réaction est variable d'une espèce animale à l'autre, elle a également été constatée avec des extraits de tissus adipeux (Goutefongea, communication personnelle).

Nous avons finalement retenu l'acide sulfanilique pour la formation du dérivé diazo avec les nitrites et la N-(Naphtyl) éthylènediamine pour la formation du dérivé coloré, ce produit étant moins cancérigène que la naphtylamine (McLoughlin, 1968). La coloration dont le maximum d'absorption se situe à 530 nm est totalement développée en 3 mn si on agite. Les lectures peuvent être réalisées

dans une plage de temps très long, au moins 24 h à condition d'impérativement placer un blanc et un standard dans la série d'échantillons pour tenir compte des variations de coloration dans le temps.

1.22. *Préparation des colonnes de cadmium*

Les colonnes de réduction sont préparées selon la méthode de GRASZHOFF 1964, consistant à réaliser un amalgame cadmium mercure à la surface de grains de cadmium. Cet amalgame est beaucoup plus efficace pour la réduction du nitrate que le cadmium métal. Par rapport à du nitrite non passé sur colonne de réduction, le nitrate est réduit entre 98 et 101 p. 100 sur nos colonnes, de plus elles sont très reproductibles. La régénération est à réaliser dès qu'une colonne s'écarte de plus de 2,5 p. 100 des valeurs standard. Pour maintenir dans le temps l'efficacité des colonnes, il est nécessaire, lorsqu'elles ne sont pas utilisées, de les régénérer toutes les 48 h en y faisant passer 2 × 20 ml H_2O, 20 ml HCl 0,1 N, 2 × 20 ml H_2O, 20 ml de tampon 9,7 et de les conserver toujours sous tampon de pH 9,7.

1.23. *Nécessité du passage sur résine*
Réduction quantitative des solutions de nitrates sur cadmium

Lorsqu'on procède au dosage des nitrates sans passage sur résine selon la technique AFNOR, on n'obtient pas une réduction quantitative de ceux-ci. Le pourcentage de réduction est fonction de la dilution de l'échantillon (tabl. 1). Il n'en est pas de même si avant la réduction des nitrates on a pris soin de purifier les extraits par passage sur résine. Le passage sur résine permet d'éliminer la fraction interférente. Cette fraction n'a pas été encore identifiée mais elle est également signalée dans d'autres extraits comme ceux des fourrages (BOUSSET-FATIANOFF et GOUET, 1971) ou même des liquides comme l'eau de mer (HIGAKI et ZEITLIN, 1962) et dans la viande (NICHOLAS et FOX, 1973). MIRNA et HOFMANN (1969) ont émis l'hypothèse que ce pourrait être les groupes SH qui joueraient ce rôle. Nous avons tenté par deux voies d'étudier ce problème. La première consistant à

TABLEAU I

Effet de la dilution de l'échantillon sur la réduction des nitrates

Dilution finale de la viande (*)	P. 100 de réduction sans passage sur colonne de résine	P. 100 de réduction avec passage sur colonne de résine
1/20	42,8	100
1/40	57,9	103,2
1/80	73,0	104,5
1/160	102,3	97,7

(*) Extrait au 1/20 de viande préparé selon la méthode AFNOR et utilisé après dilution 1/1, 1/2, 1/4, 1/8 pour préparer des solutions de standards de nitrates.

employer des composés se fixant sur les groupes SH comme le PCMB, le MMB, PMC, EMC est apparue inexploitable car ces produits inhibent la réduction même à dose très faible 10^{-10} M pour le PCMB par exemple. La seconde a consisté à ajouter des acides aminés soufrés comme la méthionine (CH_3—S—CH_2—), la cystéine (HS—CH_2—) et la cystine (—CH_2—S—S—CH_2—).

Les résultats rapportés dans le tableau 2 montrent que l'action du groupe SH est très forte avec la cystéine mais celle du groupe —S—S— de la cystine n'est peut-être pas à négliger. Nous n'avons pas pu dépasser la dose de 2 mg du fait des difficultés de mise en solution du produit au pH employé pour la réduction. Le groupe —S— de la méthionine ne semble pas avoir d'action. Des études complémentaires seraient nécessaires pour déterminer s'il y a inhibition réelle de la réduction ou si les nitrites provenant de la réduction sont immédiatement bloqués sur les groupes SH et non accessibles aux réactifs du dosage des nitrites.

TABLEAU 2

Effet de quelques acides aminés sur la réduction des nitrates

Produit		P. 100 de réduction
Témoin		100
Methionine	2 mg/l	100
	4 mg/l	103
	10 mg/l	101
Cystine	0,5 mg/l	99
	1 mg/l	100
	2 mg/l	82
Cystéine	2 mg/l	57
	4 mg/l	32
	10 mg/l	17

Dans le dosage des nitrites on obtient les mêmes résultats si l'on prépare des gammes étalon avec de l'eau ou un extrait aqueux au 1/10 de viande, il n'y a donc pas interférence des composés de l'extrait aqueux de viande lors du dosage des nitrites. Les interférences constatées lors du dosage des nitrates sont donc probablement dues uniquement à l'inhibition de la réduction.

1.24. *Étude des colonnes de résine*

Sur 4 colonnes de résine on a étudié avec une solution à 80 mg/l de NO_3K dans HCl 0,05 N la récupération dans les différentes fractions. On a fait passer successivement 10 ml de NO_3K à 80 mg/l, 3 fois 100 ml HCl 0,05 N, 50 ml NH_4OH

et 2 fois 50 ml d'eau. On recueille des fractions successives de 100 ml et l'on dose les nitrates après réduction sur cadmium.

Les résultats sont rapportés dans le tableau 3. Les colonnes ont toujours un bruit de fond représentant environ 4,75 mg de NO_3K /l, cette valeur est stable quand les colonnes sont utilisées régulièrement. De plus, cette valeur « Nitrate » est à la limite de détection de la technique analytique. On ne peut donc pas affirmer qu'il s'agit bien de nitrates qui auraient été retenus et qui seraient libérés ensuite, d'autant plus que l'on récupère quantitativement les nitrates en solution pure que l'on fait passer sur la colonne.

Les nitrites ne sont pas retenus ou sont détruits lors de leur passage sur la colonne de résine.

TABLEAU 3

Rétention et élution des nitrates sur les colonnes de résine

Fractions / Colonnes	1 10 ml NO_3 + 100 ml HCl	2 100 ml HCl	3 100 ml HCl	4 50 ml NH_4OH 50 ml H_2O	5 50 ml H_2O
1	4,89	4,89	3,35	79,44	6,98
2	4,89	3,77	4,89	83,63	6,00
3	5,17	5,45	5,03	78,88	4,75
4	4,61	3,49	2,93	76,79	4,89
$\overline{X}$	4,89	4,40	4,05	79,69	5,66
s	0,23	0,93	1,07	2,87	1,05

Absorption exprimée en D.O. × 1 000 pour 1 µg d'azote des différentes fractions obtenues lors du passage de 10 ml d'une solution de nitrate sur une colonne de résine.

Conclusions

Les réactifs proposés pour le dosage des nitrites permettent de traiter de grandes séries d'échantillons du fait de la stabilité de la coloration. On n'est jamais limité par un délai de lecture de la coloration développée si l'on incorpore dans chaque série un blanc et un standard. Bien que cela soit possible, nous ne recommandons cependant pas de reporter les lectures au lendemain du jour de la réalisation des analyses.

Pour le dosage des nitrates la purification de l'extrait par passage sur colonne de résine allonge la méthode classique mais elle permet dans tous les cas d'obtenir une réduction quantitative et reproductible. De plus, l'emploi d'échangeur d'ions permet une concentration en nitrate de l'extrait, il est donc possible dans ces conditions de doser des quantités plus faibles de nitrate.

Il est possible du fait de l'emploi des siphons, évitant le dessèchement des colonnes, de mener parallèlement autant de purifications que de réductions. Personnellement nous avons utilisé jusqu'à huit colonnes simultanément.

La méthode permet de doser les nitrites en concentration supérieure à 2,5 mg de NO_2Na/kg de produit frais avec une précision de 2 p. 100 si l'on ne tient pas compte de l'hétérogénéité d'échantillonnage. Pour les nitrates on peut doser à partir de 5 mg de NO_3K/kg de produit frais avec une précision au moins égale à 7 p. 100. L'erreur la plus importante étant introduite par la purification par passage sur résine, c'est donc ce poste analytique qu'il convient de surveiller spécialement.

II. — Proposition d'une méthode automatique de dosage des nitrites et des nitrates

2.1. *Matériel et méthode*

2.11. *Préparation des échantillons*

Les échantillons pour analyse sont traités selon la méthode AFNOR VO4 409 et VO4 410.

2.12. *Préparation des solutions*

2.121. *Tampon*

Préparer un tampon concentré 10 fois. Deux techniques sont possibles. Soit dans une fiole d'1 l placer 350 ml d'H_2O et ajouter 165,7 ml d'acide chlorhydrique à 37 p. 100 (d. 1,19). *Agiter fortement* puis verser *doucement* et en *agitant* 224,6 ml d'ammoniaque à 25 p. 100 d 0,91. Il est préférable de travailler sous hotte.

Soit dissoudre 107 g de chlorure d'ammonium dans environ 800 ml d'H_2O puis ajouter 74,9 ml d'ammoniaque à 25 p. 100 d 0,91 et ajuster à 1 000 ml.

2.122. *Solution de sulfanilamide*

Dans une fiole de 1 000 ml placer 300 ml d'H_2O, 10 g de sulfanilamide puis 500 ml d'HCl concentré ($d = 1,19$) agiter et compléter à 1 000 ml.

2.123. *Solution de I (N-naphtyl) éthylènediamine*

Dissoudre 1 g de I (N-naphtyl) éthylènediamine dans 1 000 ml d'H_2O.

2.124. *Solution de nitrite de potassium*

Réaliser une solution mère à 3,0377 g dans 1 000 ml d'H_2O puis diluer cette solution au 1/10 dans H_2O. Placer 1, 2, 3, 4 et 5 ml de cette dilution dans des fioles de 50 ml. Ajuster par H_2O, les solutions ainsi obtenues sont à 1, 2, 3, 4 et 5 µg de N du NO_2K.

2.125. *Solution de nitrate de potassium*

Réaliser une solution mère à 1,8037 g dans 1 000 ml d'H_2O (solution A) diluer A au 1/5 pour obtenir la solution B. Placer 1, 2, 3, 4 et 5 ml de solution B dans des fioles de 50 ml et ajuster par H_2O, les solutions ainsi préparées sont à 1, 2, 3, 4 et 5 µg de N du NO_3K.

2.126. *Solution de diluant*

Solution de ClNa à 0,9 p. 100 dans H_2O.

2.13. *Préparation de la colonne de réduction*

Placer 40 g de cadmium (Merck réf. 2001) dans un erlenmeyer de 250 ml, ajouter 50 ml d'HCl 2 N et agiter. Laver à l'eau distillée et décanter.

Ajouter 100 ml de solution de SO_4Cu (20 g SO_4Cu 5 H_2O/1 000 ml) agiter puis laver l'amalgame jusqu'à disparition des traces de cuivre dans les eaux de rinçage en maintenant sous eau.

Remplir sous eau une colonne de verre de 5 mm de diamètre intérieur et de longueur 5 à 30 cm.

Conditionner la colonne par passage de 750 ml de KNO_3 à 1 µg NO_3/ml soit 5 ml de solution B dans 1 000 ml auquel on ajoute 20 ml EDTA à 38 g/1 000 ml et 20 ml de tampon concentré. Rincer à l'eau distillée puis replacer la colonne sous tampon dilué 10 fois.

2.14. *Schéma de montage* (voir fig. 2)

2.2. *Observations*

Les résultats sont exprimés en densité optique multipliée par mille rapportée à un microgramme d'azote.

2.21. *Emploi de la sulfanilamide*

Dans la méthode automatique on peut utiliser ce réactif car l'addition presque simultanée, environ 10 secondes après, de I (N-naphthyl) éthylènediamine ne permet pas la formation, plus lente, du composé absorbant à 440 nm. De plus le maximum d'absorption est, avec ce couple de réactifs, à 540 nm ce qui correspond à un filtre classique Technicon (dosage de l'hémoglobine).

2.22. *Effet du chlorure de sodium*

Les résultats obtenus dans des produits ayant reçu différentes surcharges de ClNa nous ont montré que ce composé utilisé aux doses de 10 à 40 g/kg dans les charcuteries n'a pas d'influence sur la détermination du nitrite ou du nitrate, ceci confirme les résultats de Morris et Riley (1963).

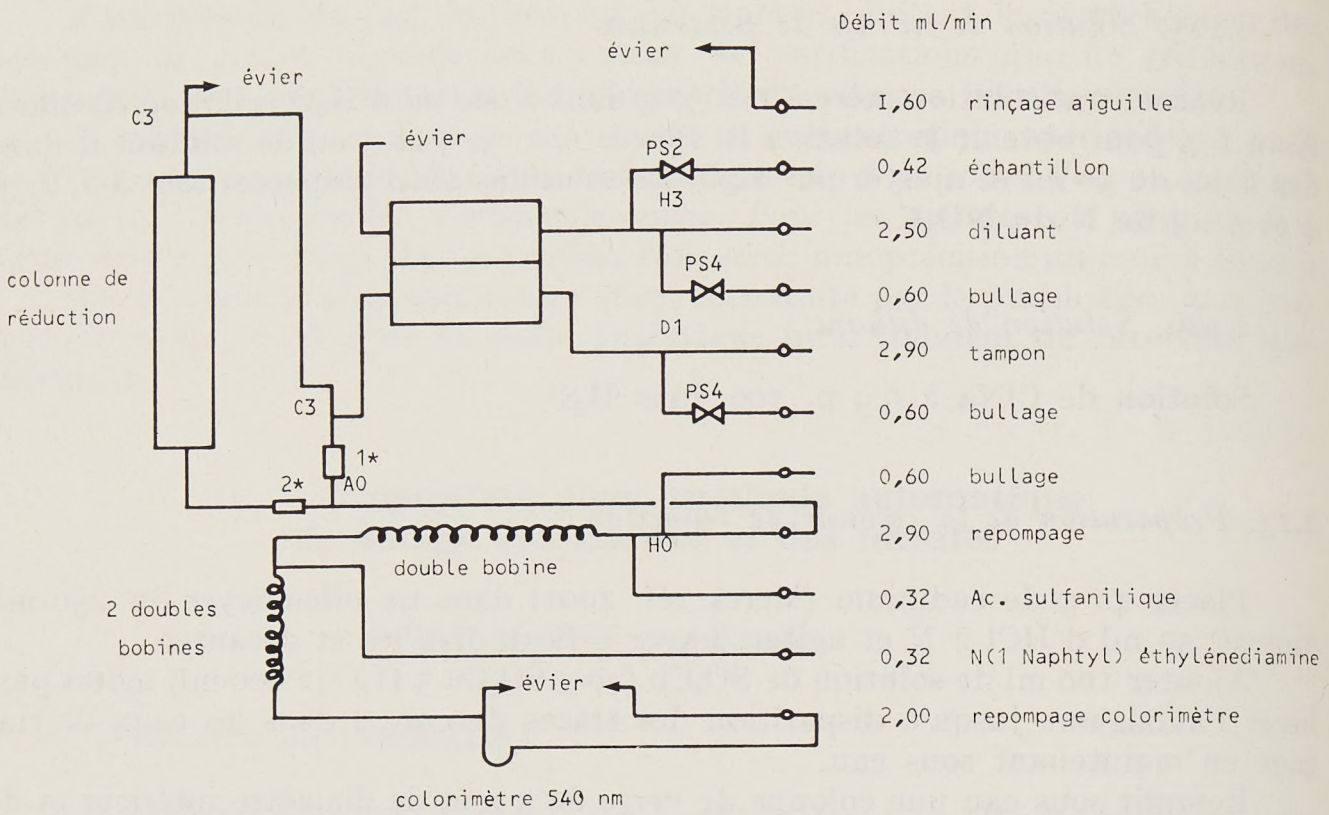

FIG. 2. — *Schéma de montage.*

(*) Tube silicone pouvant être fermé par une pince :
— pour les nitrites 1 ouvert 2 fermé;
— pour les nitrates 1 fermé 2 ouvert.

2.23. *Efficacité de la colonne de réduction*

Trois essais ont été réalisés pour tester l'efficacité de la réduction en comparant la coloration obtenue avec du nitrate par rapport à celle donnée par du nitrite. Les résultats de ces essais rapportés au tableau 4 indiquent que la réduction est toujours complète.

TABLEAU 4

Efficacité de la colonne de réduction

	D.O. X 1 000 par µg d'azote			P. 100 de réduction de NO_3	
	NO_2 direct	NO_2 « réduit »	NO_3 réduit	par rapport au NO_2 « réduit »	par rapport au NO_2 direct
1e essai	78,73 ± 2,15	74,38 ± 1,34	76,90 ± 1,81	103	98
2e essai	81,83 ± 1,43	83,23 ± 0,60	84,38 ± 1,60	101	103
3e essai	83,32 ± 2,38	81,83 ± 1,29	83,91 ± 1,02	103	100

Il est intéressant pour contrôler les analyses de connaître la valeur de l'absorption (exprimée en D.O. × 1 000) correspondant à 1 µg d'azote. Cette forme d'expression permet d'avoir les mêmes valeurs pour les nitrites et les nitrates. Pour 1 µg d'azote l'absorption doit être de 80 ± 5 dans le cas contraire il est nécessaire de vérifier l'état des tubes de pompage. Les gammes étalons étant établies en µg d'azote on doit obtenir la même densité optique que l'on détermine des nitrites ou des nitrates, ce qui permet de détecter immédiatement un éventuel défaut de la colonne de réduction.

2.24. *Effet de la température de développement de la coloration*

Le tableau 5 montre qu'il n'existe pas de différence dans les résultats obtenus pour des échantillons maintenus pendant le développement de la coloration soit à la température du laboratoire soit à 50 °C. Le développement de la coloration peut donc se faire à la température du laboratoire, les fluctuations de celle-ci seront sans aucune influence sur le dosage.

TABLEAU 5

Effet de la température de développement de la coloration

µg d'azote dans 1 ml de solution	1	2	3	4	5
Température ambiante environ 20 °C	78	76	78,3	78,5	80,8
Bain-marie 50 °C	78	76	76,6	78	79

Résultats exprimés en D.O. × 1 000 par µg d'azote.

2.25. *Effet de différents temps de développement de coloration*

Les essais sont réalisés à une cadence d'échantillonnage de 40 échantillons /h. Nous avons comparé 1 bobine de bain-marie, 2 bobines doubles et 1 bobine double. On ne constate aucune différence dans les résultats (tabl. 6). Nous avons donc retenu l'emploi de 2 bobines doubles de mélange, ce qui permet un temps de coloration pas trop important mais permet cependant d'atteindre des densités optiques élevées allant jusqu'à 2,0.

2.26. *Cadence d'échantillonnage*

La théorie voudrait que les pics obtenus dans les dosages réalisés à l'aide d'un technicon soit des courbes gaussiennes. En réalité on s'aperçoit que la courbe

TABLEAU 6

Effet de la durée de développement de la coloration

µg d'azote dans 1 ml de solution	1	2	3	4	5
1 bobine de bain-marie	78	76	78,3	78,5	80,8
2 bobines doubles	79	76,5	79	78,8	80,4
1 bobine double	81	78,5	80	80	82

Résultats exprimés en D.O. × 1 000 par µg d'azote.

enregistrée est le résultat d'une courbe sensiblement en S que l'on interrompt plus ou moins rapidement avant que la coloration ait atteint son maximum. Le maximum étant d'autant plus long à atteindre que la coloration est plus élevée, si l'on coupe la courbe trop rapidement on perd toute linéarité dans les hautes densités optiques. Si l'on veut réaliser des dosages avec une linéarité étendue sur une grande variation de concentration en produit il faut adopter une cadence de prélèvement permettant d'obtenir le maximum de coloration et dans ce cas les pics obtenus ne sont plus de forme gaussienne mais présentent un aplatissement au sommet. D'autre part des cadences plus faibles permettent d'éviter les interférences d'un échantillon sur l'autre. Nous avons retenu la cadence de 30 échantillons par heure qui permet de satisfaire aux exigences précédentes.

2.27. *Reproductibilité et linéarité*

Ces deux paramètres ont été étudiés sur des gammes étalon. La cadence d'analyse était de 30 échantillons /h. Le tableau 7 indique les résultats obtenus en ce qui concerne la reproductibilité. On peut voir que la reproductibilité est très bonne puisque le coefficient de variation est inférieur à 1 p. 100.

La linéarité de réponse est également très bonne dans la zone étudiée comme le montre la figure 3 pour le dosage directe du nitrite. Il en est de même pour le nitrite et le nitrate passés sur colonne de réduction dont les courbes correspondantes sont des droites pratiquement confondues avec celle du nitrite dosé directement. Avec le matériel Technicon que nous avons utilisé il n'est pas possible de déterminer, avec précision, des densités optiques supérieures à 1 en analyse d'échantillons, car la lecture sur l'enregistreur devient très difficile.

Vers les faibles densités, dans le dosage des nitrites le coefficient « a » (ordonnée à l'origine) n'étant pas nul il est possible que l'on perde en linéarité mais ceci doit se produire seulement pour les très faibles valeurs, car des lectures effectuées à 0,010 DO étaient encore linéaires.

TABLEAU 7

Reproductibilité des dosages

	Type de dosages		Concentration en nitrites ou nitrates en µg d'azote				
			1	2	3	4	5
D. O. lues (*)	Dosages directs des solutions de nitrites	$\overline{X}$	86,00	168,75	258,25	344,00	440,25
		Sx	0,00	0,96	0,50	2,00	0,50
	Dosages des solutions de nitrites passées sur colonne de réduction	$\overline{X}$	84,25	166,00	252,00	339,00	430,75
		Sx	0,50	0,00	0,00	2,00	0,50
	Dosages des solutions de nitrates passées sur colonne de réduction	$\overline{X}$	82,10	166,40	252,00	340,50	429,20
		Sx	0,81	0,54	1,34	2,83	0,27

Pour chaque cas $n = 4$.
(*) Résultats exprimés en D.O. × 1 000.

Nous ne traitons pas ici de la reproductibilité obtenue avec des échantillons réels, car elle intègre les reproductibilités d'extraction et de préparation des échantillons qui ne sont pas liés à la méthode analytique proprement dite. La seule constatation que l'on peut faire est que si l'on prélève soit en continu soit de façon intermittente dans le même extrait de produit à doser on obtient toujours le même résultat.

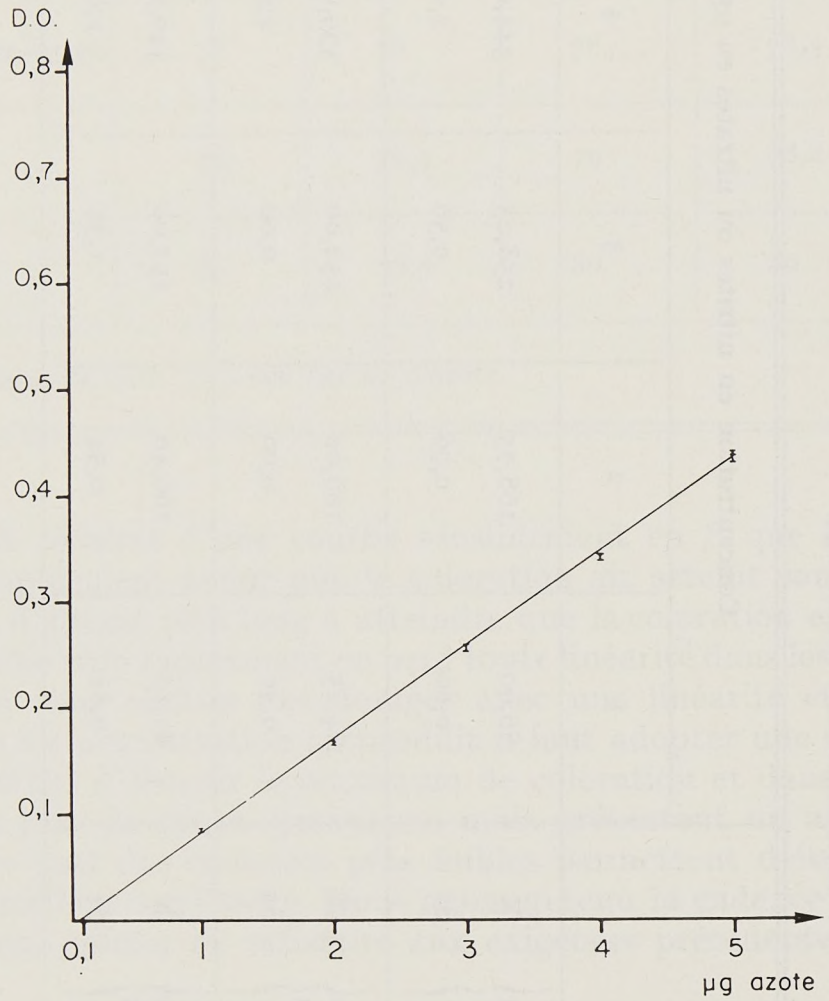

FIG. 3. — Linéarité de réponse pour les standards de nitrites. Dosage direct de solution de nitrites : $Y = -5{,}68 + 88{,}38\ X$.

2.28. *Étude de l'effet des extraits de viande sur la réduction des nitrates*

En l'absence de colonne de résine, on aurait pu craindre que des substances contenues dans la viande entraînent une diminution de l'efficacité de la colonne réductrice des nitrates comme cela est le cas pour la méthode manuelle. Pour vérifier cette hypothèse il convient de doser des solutions standard de nitrates préparées avec des extraits aqueux de viande plus ou moins dilués et de comparer ces résultats à ceux obtenus avec des standards en solution dans l'eau. Cette comparaison peut être effectuée soit de façon discontinue à partir d'un nombre limité de dilutions différentes soit de façon continue en étudiant les résultats enregistrés lors d'une dilution progressive, soit par de l'eau soit par l'extrait aqueux de viande, d'une solution concentrée de nitrates. La pompe Technicon permet de réaliser facilement cette opération en assurant en continu la variation de concentration en

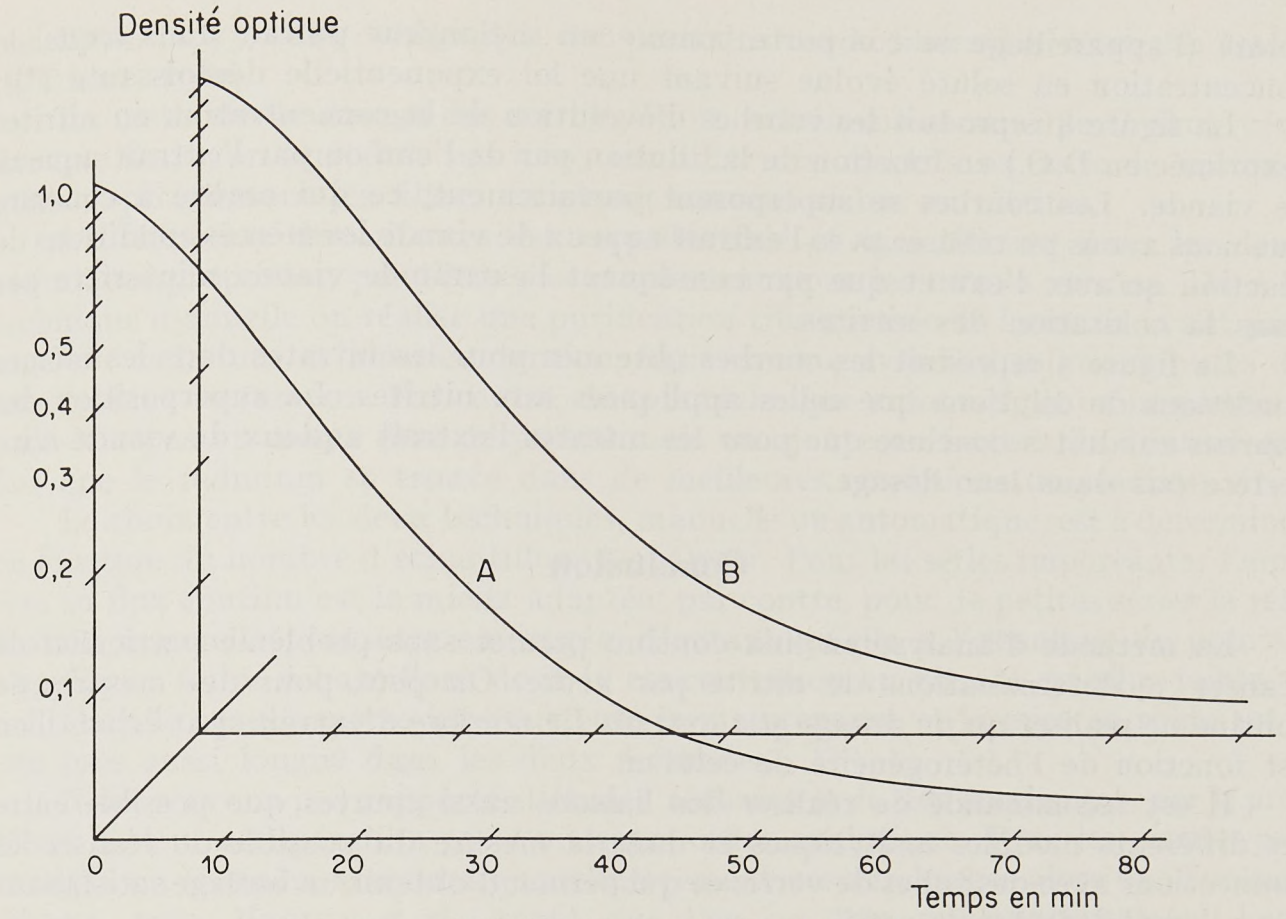

FIG. 4. — Dilution d'une solution de nitrite par :
A) de l'eau;
B) une solution de viande.

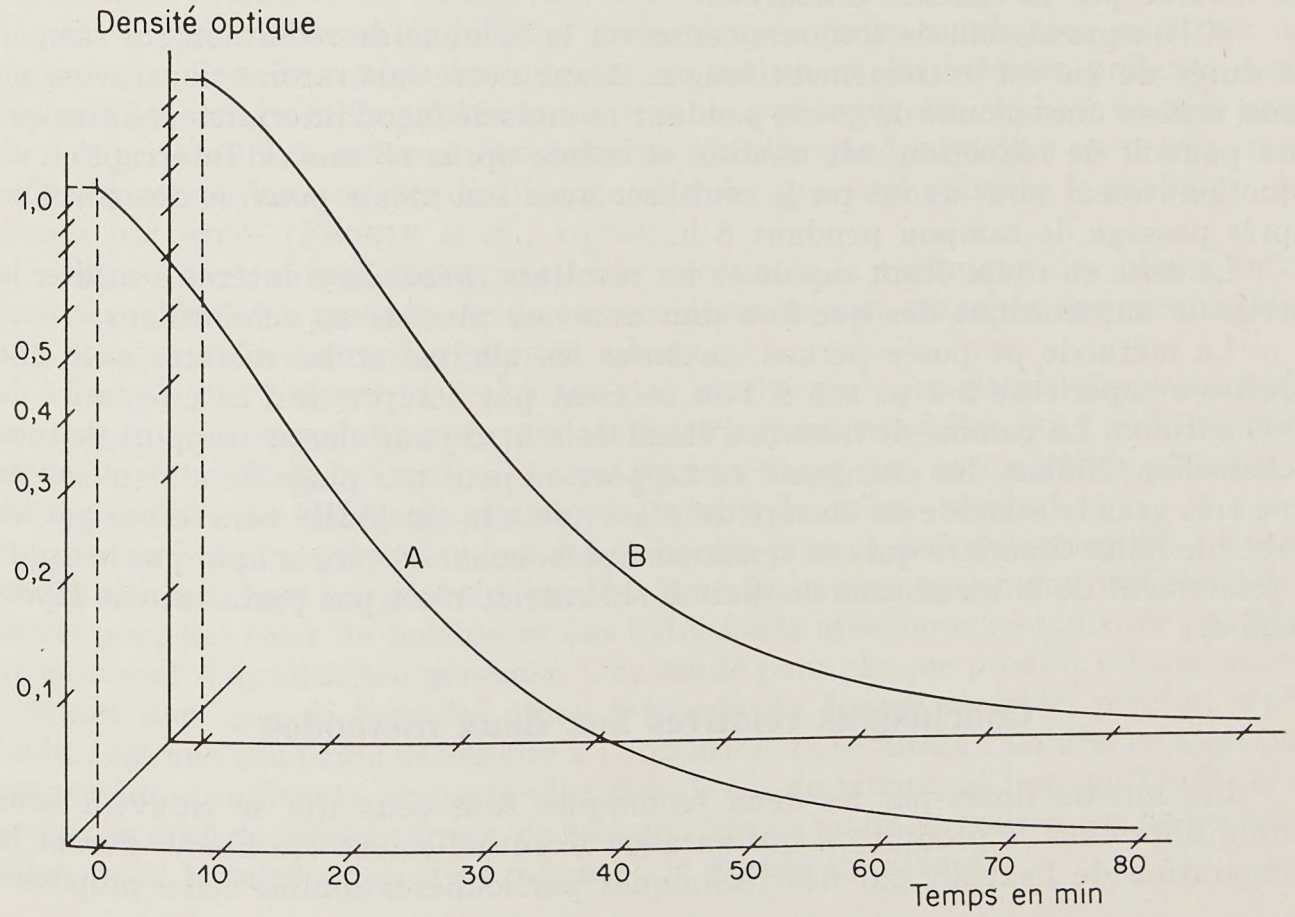

FIG. 5. — Dilution d'une solution de nitrates par :
A) de l'eau;
B) une solution de viande.

soluté (l'appareillage se comporte comme un mélangeur parfait dans lequel la concentration en soluté évolue suivant une loi exponentielle décroissante (*)).

La figure 4 reproduit les courbes d'évolution de la concentration en nitrites (exprimée en D.O.) en fonction de la dilution par de l'eau ou par l'extrait aqueux de viande. Les courbes se superposent parfaitement, ce qui amène à conclure que nous avons pu réaliser avec l'extrait aqueux de viande les mêmes conditions de réaction qu'avec l'eau et que par conséquent l'extrait de viande n'interfère pas dans la coloration des nitrites.

La figure 5 reproduit les courbes obtenues pour les nitrates dans les mêmes conditions de dilutions que celles appliquées aux nitrites. La superposition des courbes conduit à conclure que pour les nitrates l'extrait aqueux de viande n'interfère pas dans leur dosage.

Conclusion

La méthode d'analyse en flux continu permet sans problème particulier de réaliser 30 déterminations de nitrite par heure. On peut pour des mesures de routine ne réaliser qu'un dosage par extrait. Le nombre d'extraits par échantillon est fonction de l'hétérogénéité de celui-ci.

Il est recommandé de réaliser des liaisons aussi courtes que possible entre les différents modules analytiques et dans la mesure du possible de réaliser les connections avec des tubes de verre, ce qui permet d'obtenir un bullage satisfaisant sans l'emploi de tensio-actif.

Dans le schéma de montage pour les nitrates l'incorporation de la colonne de réduction allonge légèrement le temps de réponse de l'ensemble analytique mais ne modifie pas la cadence d'analyse.

Si l'on prend soin de toujours conserver la colonne de réduction sur tampon sa durée de vie est extrêmement longue. Dans notre laboratoire nous avons pu ainsi utiliser une colonne de 30 cm pendant 16 mois de façon intermittente sans que son pouvoir de réduction soit modifié et même après 18 mois d'interruption de fonctionnement nous avons pu la réutiliser avec son même pouvoir de réduction après passage de tampon pendant 8 h.

La mise en route étant rapide et les résultats précis on a intérêt à utiliser la méthode automatique dès que l'on doit analyser plus de 20 échantillons.

La méthode proposée permet de doser les nitrites et les nitrates avec une précision supérieure à 2 p. 100 si l'on ne tient pas compte de l'hétérogénéité de l'échantillon. La gamme de mesure s'étend de 1 à 10 pour chaque rapport de tube échantillon /diluant. En changeant ce rapport on peut par plage de 1 à 10 couvrir une très grande étendue de mesure de 1 à 1 500. On est limité vers le bas par les tubes de faible diamètre qui ont tendance à se boucher et vers le haut par le risque d'éclatement de la membrane de dialyse si l'extrait n'est pas parfaitement déprotéinisé.

Conclusions relatives aux deux méthodes

Les nitrites dosés par les deux techniques sont ceux qui se trouvent sous forme libre dans le produit ou qui auraient éventuellement été libérés durant la préparation de l'extrait par des techniques particulières comme celles proposées

(*) $C_0\, e^{-\frac{t}{\tau}}$ C_0 = Concentration initiale, t = temps, $\tau = \frac{V}{\dot{V}}$, V = volume du mélangeur, $\dot{V}$ = débit de renouvellement.

par MIRNA 1973, sans oublier que dans certains cas la libération n'est pas totale (BOUSSET, 1980).

La méthode de dosage des nitrites étant extrêmement sensible il faut vérifier que l'on n'en introduit pas avec les différents réactifs ou avec le matériel utilisé, spécialement les filtres en papier souvent riches en nitrites.

La réduction des nitrates est reproductible et quantitative dans les deux techniques proposées, peut-être pas exactement pour les mêmes raisons. Dans la technique manuelle on réalise une purification très poussée de l'extrait avant passage sur cadmium et ainsi ce dernier réagit comme avec des solutions pures de nitrate dans du tampon. Par contre, dans la technique automatique on ne parvient peut-être pas au même état de pureté mais on ajoute un facteur de dilution qui fait que le cadmium se trouve dans de meilleures conditions de réaction.

Le choix entre les deux techniques, manuelle ou automatique, est à déterminer en fonction du nombre d'échantillons à analyser. Pour les séries importantes l'analyse en flux continu est la mieux adaptée; par contre, pour de petites séries la réalisation manuelle peut être envisagée, d'autant qu'elle a l'avantage de pouvoir s'appliquer à des échantillons dont la concentration en nitrate est plus faible.

Dans le cas d'analyse intermittente la maintenance et la mise en route est à peu près aussi longue dans les deux méthodes.

Nous n'avons pas rapporté d'études réalisées avec des surcharges, car le problème de leur récupération est d'un autre ordre analytique. Pour les nitrites par exemple on récupère toujours quantitativement ces surcharges alors que l'on sait (MIRNA, 1973; FROUIN *et al.*, 1976) que l'on ne dose qu'imparfaitement l'ensemble des nitrites présents dans le produit analysé. L'emploi du chlorure de mercure dans les défécants peut, dans certains cas, les charcuteries cuites par exemple, permettre d'atteindre au dosage de la totalité (BOUSSET, 1980).

En ce qui concerne les nitrates nous récupérons les surcharges mais rien ne prouve que l'on dose tous les nitrates et seulement les nitrates présents dans l'échantillon analysé. Ce problème ne semble pas avoir été soulevé dans le sens du dosage total. Les nitrates étant moins réactifs que les nitrites on peut penser qu'il n'y a pas dans ce cas de blocage et donc que seul subsiste le problème du dosage par excès (FROUIN *et al.*, 1976*a*).

On ne peut actuellement pas avoir recours à la technique des bilans entre produits ajoutés et produits dosés car ces derniers sont entachés des erreurs dues aux dosages défectueux des nitrites totaux.

Dans le cas présent la technique des surcharges permettrait simplement de montrer que l'on ne perd pas de produit entre le moment où l'on fait l'addition et le moment où on lit la coloration. La méthode n'étant pas une méthode absolue le dosage réalisé sur différents standards fournit le même résultat.

Les techniques proposées ont été élaborées pour la détermination des nitrites et des nitrates dans les produits carnés. Nous les avons également utilisées avec succès pour des eaux de boisson et des baby-foods mais nous ne pouvons pas dire qu'elles sont d'application générale. Une étude pour chaque produit est nécessaire d'autant plus que, si l'emploi d'une méthode de dosage sensible, précise, répétable... est une condition nécessaire à l'obtention de résultats valables, ce n'est pas une condition suffisante, en particulier dans le cas du nitrite, où l'on sait l'influence, sur la quantité de nitrites dosée, de facteurs liés au mode de traitement de l'échantillon entre la fabrication du produit et le dosage lui même.

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Summary

Determination of nitrite and nitrate in meat products

Two methods, one manual and one automatic, were described.

Nitrites were determined by GRIESS's staining using sulfanilic acid and N-1-Naphthyl-ethylenediamine and reading at 530 nm (manual method) and sulfanilamide and N-1-Naphthyl-ethylenediamine with reading at 540 nm (automatic method).

The nitrates extracted by 0.05 N HCl were purified by the anion exchange technique using IR 45 resin to eliminate an organic fraction partly inhibiting the reduction of nitrates on a cadmium column.

The reduction column was packed with cadmium-mercury amalgam (manual method) or with cadmium-copper amalgam (automatic method).

When using the automatic method the resin treatement was replaced by a continuous dialysis.

Without resin column cystein and cystin partly inhibit nitrate reduction on cadmium column.

With manual method it was possible to determine nitrite at a minimum concentration of 2.5 mg NO_2Na /kg fresh product with an accuracy of 2 p. 100 and nitrate at a minimum concentration of 5 mg NO_3K /Kg with an accuracy of 7 p. 100. This degree of accuracy does not take into account sampling errors.

The accuracy obtained with the automatic method exceeded 2 p. 100. The range of measure was 1 to 10 for each sample /diluent ratio and 1 to 1 500 for all ratios. The threshold of detection was about 1 mg NO_2Na or NO_3K /Kg fresh products.

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Protéolyse duvant la conservation à 22 °C après irradiation gamma de muscles d'animaux axéniques (*Ratus ratus*) avec ou sans addition de nitrites ou de nitrates

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avec la collaboration technique de Thérèse MERA

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Résumé

L'irradiation γ inhibe le développement bactérien et peut donc être utilisée comme technique de conservation des denrées alimentaires. Par contre, son rôle sur les enzymes protéolytiques est encore discuté. En employant de la chair d'animaux axéniques, nous mettant ainsi à l'abri de toutes actions bactériennes, nous avons étudié l'effet de l'irradiation à 10-30-50 KGy sur la protéolyse durant la conservation.

Durant l'irradiation à 0 °C l'azote soluble (Ns) en p. 100 de l'azote total (Nt) diminue de 27 p. 100 à 24 p. 100, alors que l'azote non précipitable par le T.C.A. (Nnp) en p. 100 du Nt demeure constant.

Lors de la conservation pendant 4 semaines à 22 °C le Ns p. 100 Nt augmente de 25,5 p. 100 à 33,2 p. 100 et le Nnp augmente lui de 10,8 p. 100 à 25,8 p. 100, quelle que soit la dose d'irradiation.

La présence de nitrite ou de nitrate à des doses de 150 et 300 ppm ne modifie pas sensiblement le phénomène.

Dans nos conditions jusqu'à 50 KGy l'irradiation n'a pas d'effet favorisant ou inhibiteur sur la protéolyse globale.

Pour la conservation des denrées alimentaires, spécialement les viandes fraîches ou les viandes saumurées, on devra associer au traitement de stérilisation γ un processus thermique pour inactiver les protéases.

Mots clés : *protéolyse, irradiation gamma, conservation de muscle, rat, animaux axéniques.*

Introduction

L'irradiation γ qui inhibe le développement microbien peut être une méthode de conservation des denrées alimentaires. Ses effets propres sur la stabilisation des réactions de dégradation qui se produisent normalement dans les produits doivent cependant être précisés pour tirer le meilleur parti de cette technique. Cette remar-

que concerne particulièrement les viandes qui peuvent subir, avec le temps, une protéolyse endogène assez marquée.

L'effet de l'irradiation sur la protéolyse *post mortem* à long terme des muscles est encore mal éclairci.

Une étude bibliographique de ce problème fait rapidement apparaître la diversité des résultats obtenus. Pour certains auteurs (Crake *et al.*, 1957; Bautista F. R., Thompson, Cain, 1961; Cain *et al.*, 1968, Urbain, 1978) l'irradiation n'entraîne pas d'inhibition de la protéolyse. Par contre pour Doty, Wachter (1955), Shults, Cohen, Wierbicki (1975), Taub *et al.* (1979), il y a inhibition au moins partielle.

De toute évidence cette dernière ne peut être totale, car des auteurs comme Drake *et al.* (1957), Cain *et al.* (1958), ont montré l'apparition de cristaux de tyrosine dans les produits conservés assez longtemps à température élevée. Une partie du désaccord peut être dû au fait que certains auteurs étudient la protéolyse *in situ* dans le milieu étudié alors que d'autres étudient l'activité des protéases, après leur extraction, sur des substrats divers; Giovannozzi *et al.* (1968) ont montré, par exemple, que pour des hydrolases l'inactivation des enzymes était d'autant plus forte que l'irradiation était réalisée à un stade plus avancé de la purification. Rhodes (1968) a de même montré que l'inactivation croissait avec la dilution de l'enzyme soumise au traitement. Sur des protéases de foie, cet auteur ne trouve une inhibition que de 85 p. 100 pour 400 KGy. A 50 KGy qui constitue la dose de stérilisation il semble donc bien que la protéolyse ne peut pas être totalement inhibée.

Pour étudier l'influence de l'irradiation sur la viande il n'est pas possible en fait d'utiliser des viandes classiques. En effet, si celles-ci sont paucibactériennes au moment de l'abattage, elles sont très rapidement contaminées et l'effet des germes bactériens sur la viande interfèrera avec la protéolyse endogène. Le traitement d'irradiation réduira d'autant plus la flore microbienne que la dose utilisée sera plus forte, mais les enzymes bactériennes des germes détruits peuvent être préservées. D'autre part, si la dose d'irradiation utilisée est faible, le développement bactérien reste notable et son action protéolytique peut ne pas être négligeable.

De fait, pour s'affranchir des actions des enzymes bactériennes dans l'étude de l'effet de l'irradiation sur la protéolyse, il faut mettre en œuvre, au départ, un produit parfaitement stérile.

La présente étude rapporte les résultats obtenus en utilisant de la chair de rats axéniques pour étudier la protéolyse globale après irradiation à différentes doses. Ce type d'animal connu comme possédant un équipement protéolytique musculaire apparemment plus développé que celui de la plupart des animaux a été retenu non pour cette raison mais pour la facilité d'obtention, dans cette espèce, d'animaux axéniques.

Des essais de même nature ont été aussi conduits sur des viandes après addition de nitrites et de nitrates pour voir l'influence éventuelle sur la protéolyse après irradiation de ces additifs fréquemment utilisés en technologie carnée.

Matériel et méthode

L'ensemble des manipulations de préparation des échantillons est réalisé dans l'isolateur stérile dans lequel ont été élevés les animaux.

Des rats Fisher âgés de 9 mois à 1 an sont sacrifiés par anesthésie poussée à

l'éther puis immédiatement saignés par section des veines et artères jugulaires Après dissection, la chair récupérée est coupée à l'aide de ciseaux en morceaux qu'on mélange. L'ensemble ainsi obtenu est réparti au hasard dans des flacons de verre contenant chacun environ 20 g. Les flacons sont bouchés hermétiquement et sortis de l'isolateur. Après une nuit au réfrigérateur à 0-2 °C l'irradiation est réalisée au CAPRI (*). Le rayonnement γ est fourni par des barres de Cobalt 60 placées en piscine. Les échantillons sont enfermés dans un conteneur rempli de glace qui maintient la température au voisinage de 0 °C. Les doses appliquées ont été de 10-30-50 KGy au débit de dose de 37 KGy par heure.

Après irradiation une partie des échantillons est analysée immédiatement et une autre conservée pendant 4 semaines à 22 °C.

Lors de l'ouverture des flacons il est procédé à un examen bactériologique par observation directe et ensemencement pour tester la stérilité. L'ensemble de l'échantillon est finement broyé au broyeur à couteaux jusqu'à obtention d'une pâte homogène.

L'azote total (N*t*) est déterminé sur ce broyat.

Une aliquote est extraite dans l'eau au 1/10 par trois homogénéisations de 1 mn à l'Ultraturax. L'homogénéisat est centrifugé à 10 000 g pendant 10 mn à 2 °C et le surnageant immédiatement séparé. L'azote dosé dans le surnageant correspond à l'azote soluble (N*s*) du broyat. Dix ml de ce surnageant additionnés de 10 ml de TCA à 10 p. 100 sont placés au réfrigérateur une nuit à 2 °C. Après centrifugation à 10 000 g 10 mn à 2 °C l'azote dosé dans ce nouveau surnageant représente l'azote non précipitable par le TCA (N*np*).

Tous les dosages d'azote sont réalisés par microdistillation après minéralisation KJELDAHL. L'azote soluble et l'azote non précipitable par le TCA sont exprimés en pourcentage de l'azote total. L'azote non précipitable par le TCA est également exprimé en pourcentage de l'azote soluble.

Des essais ont également été réalisés sur des produits irradiés en présence de nitrite de potassium à la dose de 150 et 300 ppm et de nitrate de potassium à la dose de 150 ppm.

Les analyses sont réalisées en double sur deux échantillons pour chaque point expérimental.

Pour obtenir une répartition homogène du nitrite et du nitrate de potassium dans les essais de protéolyse ces produits sont ajoutés en solution aqueuse. Après conservation le contenu des flacons, qui ne peut pas être broyé de façon satisfaisante, est lyophylisé puis broyé.

L'azote total est déterminé sur le produit sec.

Une aliquote est extraite dans l'eau au 3/100 par trois homogénéisations de 1 mn à l'Ultraturax, l'homogénéisat est ensuite traité comme celui obtenu à partir des échantillons frais.

Résultats et discussion

Pour l'étude des phénomènes considérés ici nous appellerons, par convention, solubilisation la variation du rapport $\frac{Ns}{Nt}$ alors que la protéolyse est définie par la variation du rapport $\frac{Nnp}{Nt}$.

(*) CAPRI : Centre d'Application et de Promotion des Radiations Ionisantes Saclay, 91400 Orsay.

1. — *Protéolyse sans addition de nitrite ou de nitrate*

A) *Effet de l'irradiation*

Lors de l'irradiation il se produit une légère insolubilisation de l'azote qui est d'autant plus forte que la dose est plus élevée (fig. 1). L'azote soluble décroît de 27 p. 100 à 24 p. 100 de l'azote total lorsque total l'on passe de 0 à 50 KGy, soit 11 p. 100 de variation relative. Ces résultats confirment ceux de LAWRIE (1961) et

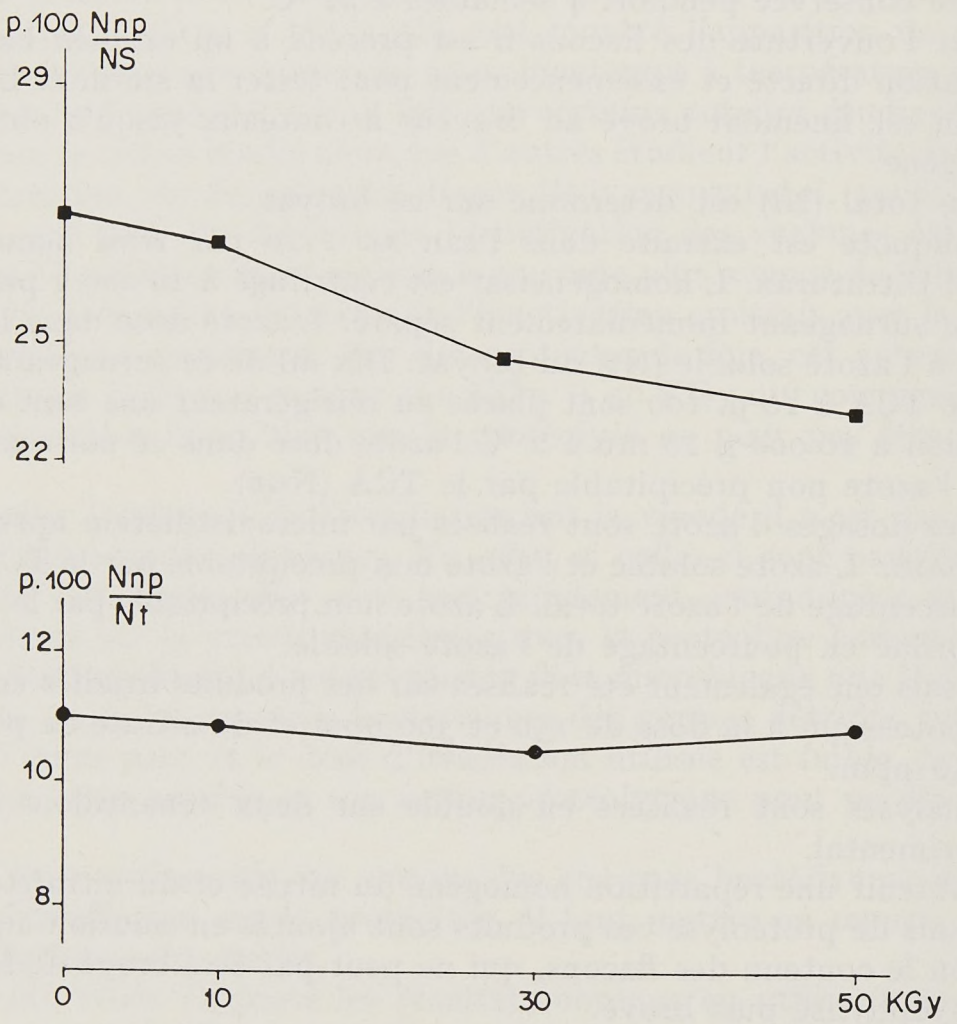

FIG. 1. — *Variation des fractions azotées durant l'irradiation en fonction de la dose.*
——■—— Azote soluble (Ns) en pourcentage de l'azote total (Nt).
——●—— Azote non précipitable au TCA (Nnp) en pourcentage de l'azote total (Ns).

NAKAI (1974) qui avaient déjà montré cette diminution des protéines solubles. Cette insolubilisation pourrait être due à une agrégation des protéines comme le constate DIEHL (1979) sur l'hémoglobine. L'azote non précipitable au TCA en p. 100 de l'azote total demeure pratiquement inchangé.

B) *Évolution durant la conservation suivant l'irradiation*

Sur une période de 4 semaines l'azote soluble s'accroît de 25,5 p. 100 à 33,2 p. 100 de l'azote total, soit une augmentation de 30 p. 100. Il n'y a pas d'effet de la dose d'irradiation comme le montre la figure 2 et comme l'indique l'étude sta-

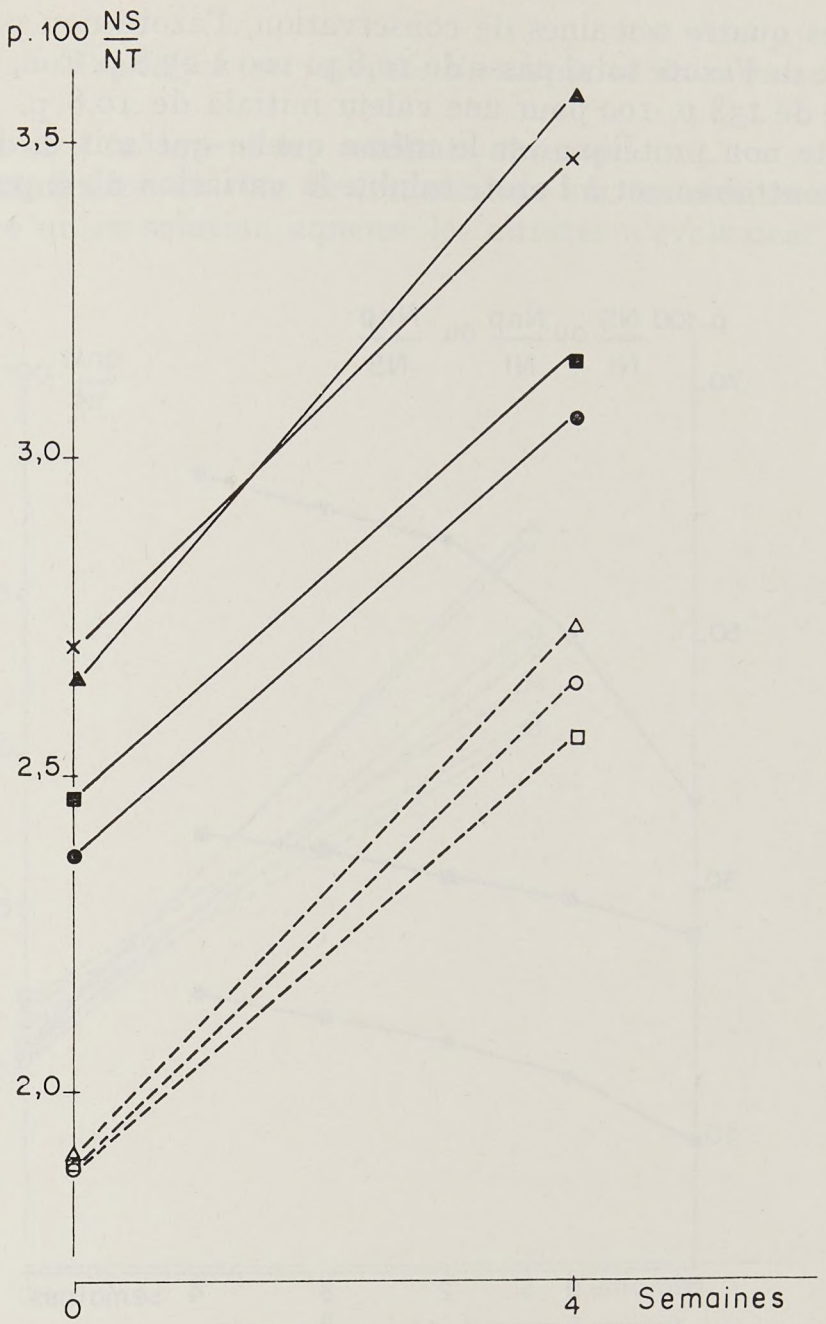

FIG. 2. — *Évolution de l'azote soluble (Ns) en pourcentage de l'azote total (Nt) durant la conservation.*

Viande seule :
—×— Témoin.
—▲— 10 KGy.
—■— 30 KGy.
—●— 50 KGy.

En présence de nitrite ou de nitrate de potassium :
..... △ ..... 10 KGy.
..... □ ..... 30 KGy.
..... ○ ..... 50 KGy.

tistique qui fait ressortir une différence significative des ordonnées à l'origine mais non des pentes. Nous ne retrouvons pas les résultats de BAUTISTA (1961) qui non seulement n'enregistrait pas une inhibition due à l'irradiation mais constatait une augmentation plus rapide de la solubilisation et de la protéolyse de la fraction solubilisée après le traitement. Dans des essais préliminaires réalisés sur d'autres rats de même type nous avons constaté que la solubilisation de l'azote était linéaire en fonction du temps (fig. 3).

Durant les quatre semaines de conservation, l'azote non protéique exprimé en pourcentage de l'azote total passe de 10,8 p. 100 à 25,8 p. 100, soit une augmentation relative de 138 p. 100 pour une valeur initiale de 10,8 p. 100. L'accroissement de l'azote non protéique est le même quelle que soit la dose d'irradiation (fig. 4). Mais contrairement à l'azote soluble la variation n'est pas constante dans

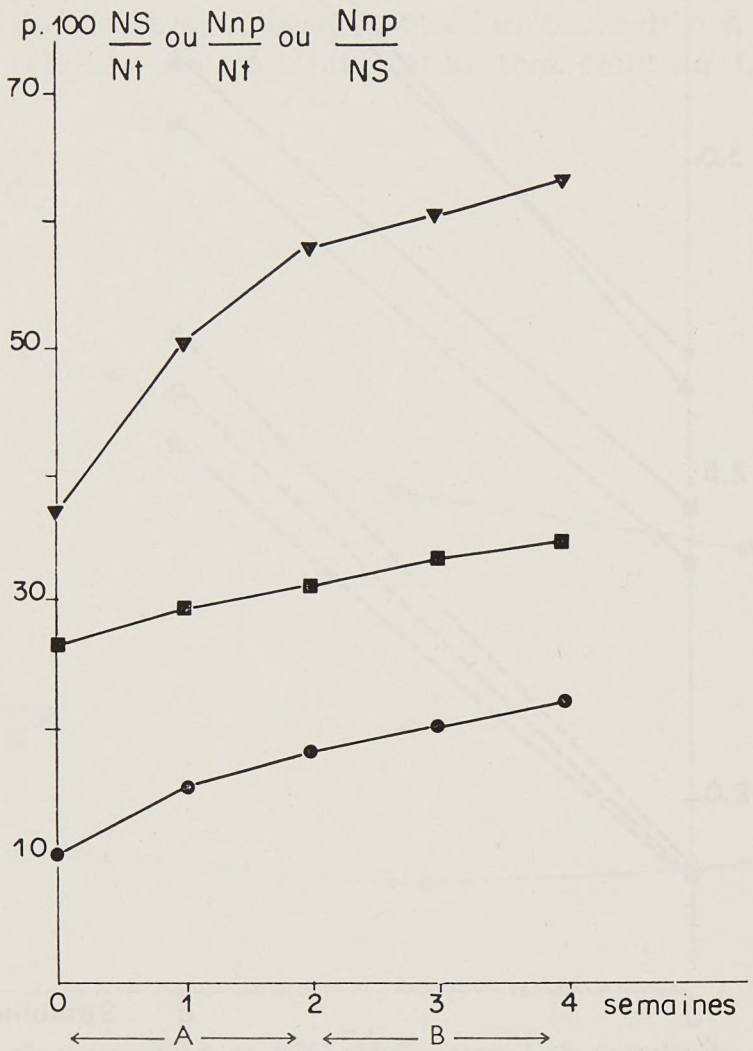

FIG. 3. — *Évolution des fractions azotées durant la conservation.*

——■—— Azote soluble (Ns) en pourcentage de l'azote total (Nt).
——●—— Azote non précipitable au TCA (Nnp) en pourcentage de l'azote total.
——▾—— Azote non précipitable au TCA en pourcentage de l'azote soluble.

le temps, rapide au début, elle se ralentit ensuite pour devenir sensiblement linéaire (fig. 3).

Pour expliquer ce phénomène il est intéressant d'étudier la proportion d'azote non précipitable par le TCA dans l'azote soluble. On constate que dans la quasi totalité des essais que nous avons réalisés cette proportion ne dépasse pas 85 p. 100. Dans la première phase de la conservation (A — fig. 3) la protéolyse intéresse les fractions azotées solubles existant au temps zéro et celles qui sont solubilisées pendant cette période, alors qu'ensuite elle n'intervient que sur les fractions azotées qui sont progressivement solubilisées. Le phénomène global d'apparition de l'azote non protéique semble alors se ralentir (B — fig. 3).

2. — *Protéolyse en présence de nitrite et de nitrate*

Les figures 2 et 4 indiquent les résultats obtenus.

Un essai préliminaire du rôle de l'irradiation sur les nitrites et les nitrates nous avait montré qu'en solution aqueuse les nitrates n'évoluaient pas alors que

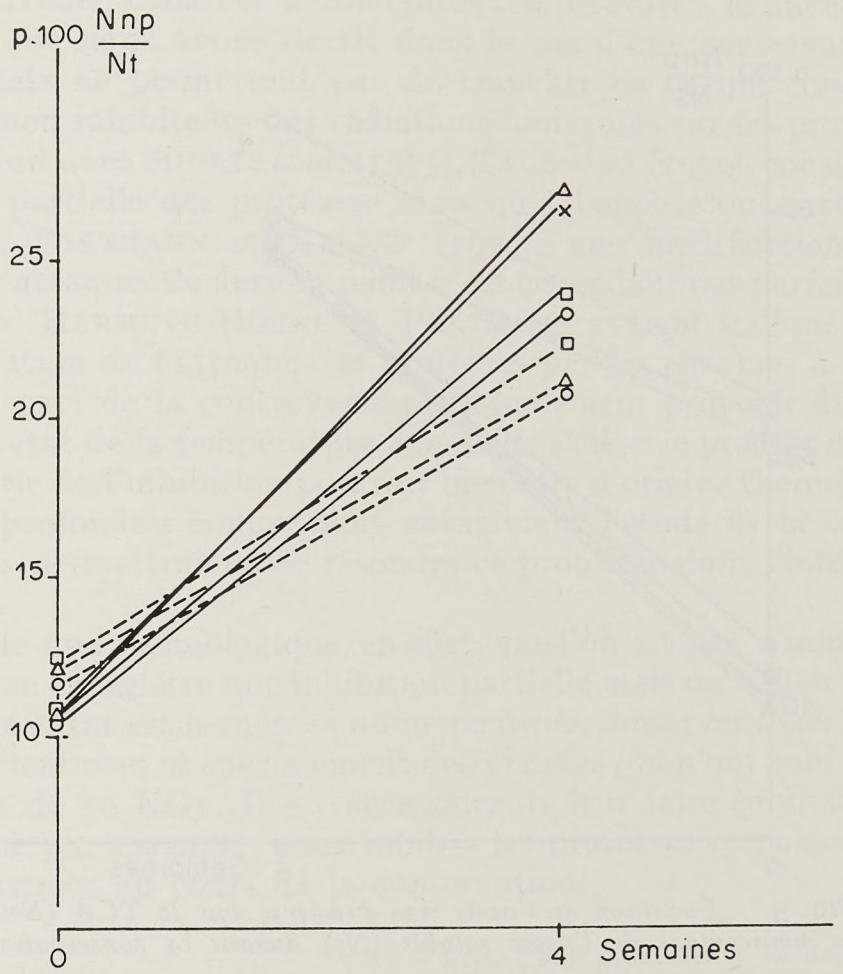

FIG. 4. — *Évolution de l'azote non précipité par le TCA (Nnp) en pourcentage de l'azote total (N) durant la conservation.*

Viande seule :

——×—— Témoin.
——▲—— 10 KGy.
——■—— 30 KGy.
——o—— 50 KGy.

En présence de nitrite ou de nitrate de potassium :

..... △ ..... 10 KGy.
..... □ ..... 30 KGy.
..... o ..... 50 KGy.

les nitrites étaient oxydés en nitrates ce qui correspond à l'action classique des rayonnements ionisants sur les réducteurs en solution aqueuse. En présence d'un extrait aqueux au 1/10 de viande, par contre, une partie importante de nitrites n'était plus dosée sans que l'on note pour autant une augmentation des nitrates. Ce fait pourrait s'expliquer par un blocage des nitrites sur les protéines du milieu.

La viande mise en œuvre dans ces essais de protéolyse est apparemment plus

pauvre en azote soluble alors qu'elle a sensiblement la même constitution en azote non précipitable au TCA.

La plus faible extraction de l'azote soluble dans ces produits s'explique peut-être par une mauvaise réhydratation du produit.

Compte tenu des deux remarques précédentes, l'examen des courbes de la figure 2 fait ressortir que la solubilisation de l'azote durant la conservation est sensiblement la même en valeur absolue en présence et en absence de nitrite ou

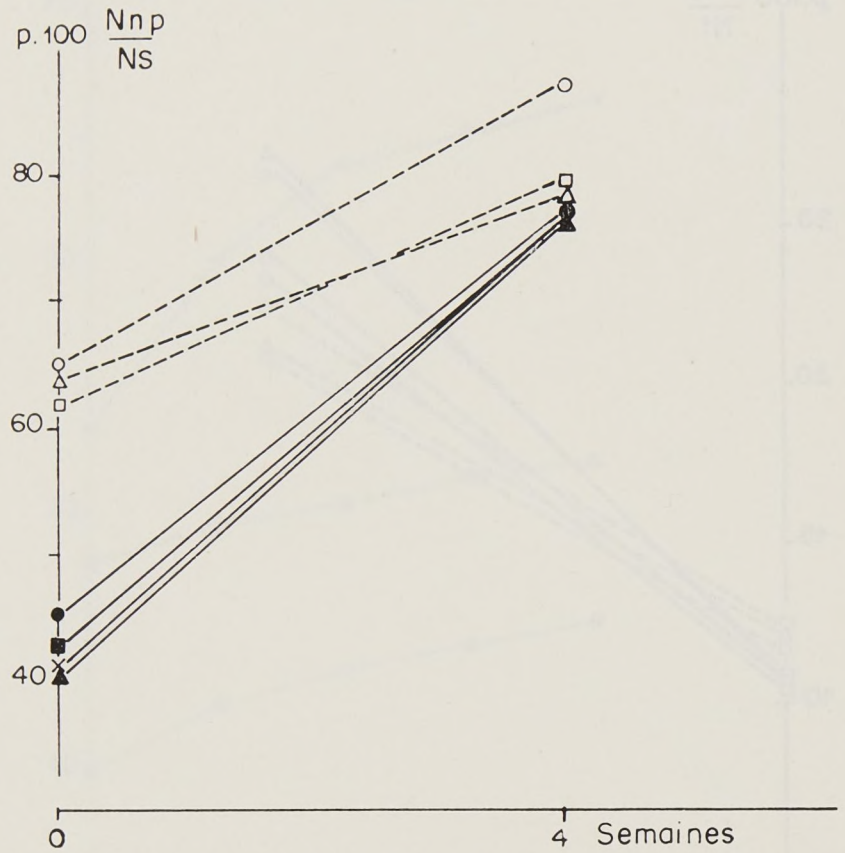

FIG. 5. *Évolution de l'azote non précipité par le TCA (Nnp) en pourcentage de l'azote soluble (Ns) durant la conservation.*

Viande seule :

——×—— Témoin.
——▲—— 10 KGy.
——■—— 30 KGy.
——●—— 50 KGy.

En présence de Nitrite ou de Nitrate de potasisum :

..... △ ..... 10 KGy.
..... □ ..... 30 KGy.
..... ○ ..... 50 KGy.

de nitrate alors qu'on aurait pu s'attendre à une solubilisation plus forte du fait que d'après RHODES (1968) ces produits joueraient un rôle de « protection » des enzymes.

L'augmentation de l'azote non précipitable au TCA est légèrement moins rapide en présence de NO_2K (fig. 4). Cette forme d'azote représente après 4 semaines de conservation des valeurs de 79 à 87 p. 100 de l'azote soluble total (fig. 5) soit sensiblement un même ordre de grandeur que celui qui est observé en l'absence de nitrite ou de nitrate.

Conclusion

Dans nos conditions expérimentales l'irradiation jusqu'à 50 KGy ne semble pas avoir d'action ni inhibitrice ni favorisante sur la protéolyse.

L'intensité de la protéolyse du muscle normal, non irradié, double tous les 10°, entre 2 et 32 °C (Bousset 1980) et il serait intéressant de vérifier si, dans le cas de muscle irradié, conservé à différentes températures, le phénomène est semblable à celui que nous avons décrit dans le cas d'une conservation à 22 °C.

Nos résultats ne permettent pas de trancher en ce qui concerne les effets inhibiteurs ou non inhibiteurs des radiations ionisantes sur les protéases. En effet on peut, en accord avec Shults *et al.* (1975), Taub *et al.* (1979), considérer que l'on a une inhibition partielle des protéases mais qu'à l'opposé on a, suivant Paul et Kumta (1973), Partmann et Keskin (1979), une modification des protéines qui facilite leur attaque. Ce dernier point n'est cependant pas parfaitement démontré car en 1976, Harmuth-Hoene et Partmann avaient indiqué qu'il n'y avait pas de modification de l'attaque des protéines par les enzymes à la suite de l'irradiation. Une part de la controverse peut également provenir du fait que rarement il est fait état de la température à laquelle s'élève le produit durant l'irradiation et une partie de l'inhibition pourrait bien être d'origine thermique. Seules des études plus approfondies comprenant notamment l'étude de la composition des fractions isolées permettraient de résoudre ce problème dont l'intérêt est uniquement théorique.

Du point de vue technologique, en effet, que l'on ait pas d'inhibition des protéases ou que l'on enregistre une inhibition partielle mais un milieu plus facilement attaquable, le résultat est le même : on ne peut pas conserver dans leur état initial des denrées alimentaires et spécialement des viandes qui n'ont subi qu'une irradiation stérilisante de 50 KGy. Il est nécessaire de leur faire subir un autre traitement, thermique par exemple, pour inhiber les protéases responsables des modifications enregistrées au cours de la conservation.

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Remerciements

Nous remercions le Laboratoire des animaux sans germes du C.N.R.S. (Dr. Doct. Saquet) pour la fourniture gracieuse des animaux nécessaires à cette expérimentaiion et l'aide technique apportée lors des manipulations en isolateurs.

Summary

Proteolyses during the storage at 22 °C after gamma irradiation of axenic animal muscle (Ratus ratus) *with or without nitrite or nitrate addition*

Gamma irradiation inhibits the bacterial development and may thus be used as a food preservation technique. However its effect on proteolytic enzymes is still discussed. Using axenic animal muscle, thus avoiding any bacterial action, we studied the effect of γ irradiation at 10-30-50 KGy on the proteolysis during storage.

During irradiation at O °C, soluble nitrogen (Ns) in p. 100 of total nitrogen (Nt) regressed from 27 to 24 p. 100, whereas T.C.A. non précipitable nitrogen (Nnp) in p. 100 of (Nt) remained constant.

During a storage period of 4 weeks, at 22 °C, Ns p. 100 Nt increased from 25.5 to 33.2 p. 100 and Nnp from 10.8 to 25.8 p. 100 whatever the irradiation dose.

The presence of nitrite or nitrate both at doses of 150 and 300 ppm does not noticeably change the phenomenon.

In our conditions, an irradiation up to 50 KGy has no favorable or inhibitory effect on the overall proteolysis.

For preservation of food, especially fresh meat or brined meat, a heat treatment should be combined with the γ irradiation to inactivate the proteases.

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Tannage par le formol des aliments protéiques du ruminant

II. — Effet du taux de formol et du mode de traitement sur quelques caractéristiques de la fraction azotée

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avec la collaboration technique de Françoise KOZLOWSKI

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Résumé

Les teneurs en formol extractible (Fe) ou libérable par hydrolyse (Fh) ainsi que la solubilité azotée et la désamination en « rumen artificiel » ont été mesurées sur 7 produits tannés dans des conditions standardisées à l'aide de doses croissantes de formol. Dans les limites du présent essai, il existe une relation linéaire entre taux de formol mis en jeu et teneurs en Fe ou Fh; cette relation dépend de la nature des produits mais ne semble pas liée de manière évidente à leur composition biochimique. Entre la solubilité azotée et la désamination en « rumen artificiel », il existe une relation étroite. Cette dernière est améliorée par prise en compte de la digestibilité de la matière organique. On en déduit qu'environ 20 p. 100 de l'azote insoluble des protéines protégées par tannage sont dégradables dans le rumen. Le traitement à la vapeur, accompagnant la granulation, provoque une libération partielle du formol fixé et une augmentation de la solubilité azotée. La présence, dans un mélange d'aliments, d'un composant formolé ayant subi une durée de maturation suffisante ne semble pas avoir d'influence marquée sur les caractéristiques de la fraction non traitée.

Mots clés : *ruminants, protéines, solubilité, tannage, formaldehyde.*

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1. — Introduction

Le tannage par le formaldehyde des protéines alimentaires du ruminant réduit leur désamination sous l'action des microorganismes du rumen; s'il est bien conduit, il n'altère pas notablement leur digestion dans la suite du tractus digestif (ZELTER, LEROY et TISSIER, 1970). Ce traitement a trouvé en France une application industrielle depuis 1976 sur les tourteaux de soja et de colza.

Sur le plan expérimental, de très nombreuses études ont été réalisées, tant *in vitro* (PETER *et al.*, 1971; CAJA *et al.*, 1977) qu'*in vivo* sur des aliments protéiques très divers : caséine (FAICHNEY et WESTON, 1971; MILLS *et al.*, 1972; SHARMA, INGALLS et PARKER, 1974; WACHIRA *et al.*, 1974; WILLIAMS et SMITH, 1975; STOBBS, MINSON et MCLEOD, 1977); lait écrémé (DELORT-LAVAL, LEROY et ZELTER, 1972) protéines de lactosérum (RODRIGUEZ, MULLER et SCHINGOETHE, 1974), tourteaux de tournesol (AMOS, BURDICK et HUBER, 1974; CAJA *et al.*, 1977), d'arachide (MILLER, 1972; FAICHNEY, 1972), de colza (SHARMA, INGALLS et PARKER, 1974); et de soja (PETER *et al.*, 1971; NIMRICK, PETER et HATFIELD, 1972; WACHIRA *et al.*, 1974; AMOS, BURDICK et HUBER, 1974; NISHIMUTA, ELY et BOLING, 1974; CAJA *et al.*, 1977; THOMAS, TRENKLE et BURROUGHS, 1979), fèverole et pois (PELLETIER et BOUCHARD, 1978). Plusieurs essais ont également été menés sur des mélanges d'aliments traités par le formol (WACHIRA *et al.*, 1974; FERGUSON, HEMSLEY et REIS, 1977; VÉRITÉ *et al.*, 1977).

Ces résultats bibliographiques sont très hétérogènes; nombre d'auteurs ne précisent pas suffisamment les conditions de traitement de leurs aliments expérimentaux. Or, le procédé de traitement, le taux de formol appliqué sont des facteurs importants de la qualité des produits. De plus, la marge est étroite, qui sépare un aliment insuffisamment tanné et donc protégé partiellement contre l'action des microorganismes du rumen et les protéines devenues, du fait d'un traitement excessif, inaccessibles à l'action des enzymes digestives. C'est donc pour préciser, sur une série plus étendue d'aliments protéiques, les conditions optimum de tannage par le formol que, dans le présent travail, les mesures de solubilité azotée en milieu tampon et de désamination des aliments *in vitro* en présence de microorganismes du rumen ont été complétées par le dosage du formol sous ses différentes formes. Il est rappelé, à cet égard, (TOME *et al.*, 1979) que le formol, dans un aliment traité, peut être caractérisé sous deux états : le formol extractible (Fe), libre ou faiblement fixé, et le formol libéré par hydrolyse (Fh), qui inclut la somme du formol n'ayant pas réagi et de celui impliqué dans des liaisons réversibles; la différence (Fh — Fe) représente la quantité de formol réversiblement liée au substrat (Fr) et fournit une estimation de l'intensité du traitement. C'est par ces méthodes qu'ont été évalués, sur 7 aliments protéiques, l'influence des paramètres du tannage (taux de formol mis en jeu, concentration de la solution d'agent tannant) et, sur des aliments formolés au laboratoire ou dans l'industrie, le rôle des traitements ultérieurs tels que la durée du stockage, le mélange à des aliments non traités, l'action de la vapeur ou l'agglomération.

2. — Matériels et techniques

2.1. — *Substrats*

Les sources protéiques soumises aux essais de laboratoire, leurs teneurs en matière sèche et en protéines brutes, exprimées en pour cent du produit tel quel, sont respectivement les suivantes :

— Tourteau d'arachide d'extraction (94,1; 50,2);
— Tourteau de tournesol décortiqué d'extraction (91,1; 38,4);
— Tourteau de coton d'extraction (89,4; 40,5);

— Pois d'hiver, variété « FRIMAS » (87,1; 23,2);
— Fèverole d'hiver, variété « SORAVI » (87,1; 25,6);
— Lupin blanc (93,6; 29,3);
— Protéines de lactosérum doux, purifiées par ultrafiltration (92,6; 69,0).

2.2. — *Traitements*

2.21. — *Méthode de tannage*

Dans la méthode de référence (ZELTER, LEROY et TISSIER, 1970), les substrats sont broyés au broyeur à marteaux (grille à trous de 1 mm) et homogénéisés. Cinquante grammes d'échantillon sont mélangés avec des solutions de formaldéhyde apportant 0,0; 0,2; 0,4; 0,6; 1; 1,2 de formaldéhyde pur p. 100 de substrat à traiter (p /p). Les solutions sont suffisamment diluées pour former une pâte homogène avec le substrat : un essai préliminaire a permis de déterminer les quantités de liquide à ajouter à cet effet soit, pour 50 g d'échantillon : tourteau d'arachide et de tournesol, 150 ml; tourteau de coton, pois et fèverole, 70 ml; lupin, 100 ml; protéines de lactosérum, 50 ml. Les aliments mélangés à la solution de formol sont laissés au repos pendant 24 heures à température ambiante, puis lyophylisés. Pour tester l'effet du temps de réaction sur des échantillons de pois, ceux-ci sont traités par 0,8 p. 100 (p /p) de formol pur et laissés en contact avec la solution pendant des durées variables (entre 24 h et 8 j) avant lyophylisation.

Un autre traitement qui diffère sensiblement de la méthode de référence et qui est plus proche de la réalité industrielle (DELORT-LAVAL, 1980) a également été mis en œuvre. Il consiste à ajouter par pulvérisation une solution concentrée de formol à de petites quantités d'aliment placées dans un ballon tournant. La quantité de formol pulvérisée en une dizaine de minutes est déterminée par pesée du flacon d'agent tannant avant et après le traitement. Dans la présente étude, l'essai a porté sur 50 g de pois, traités par une solution commerciale de formol à 32,4 p. 100 (p /v).

2.22. — *Traitements après tannage*

Des essais de mélange et d'agglomération ont porté sur des tourteaux de soja et de colza tannés respectivement à 0,3 et 0,4 p. 100 de formol dans une installation industrielle dont le principe a été sommairement décrit ailleurs (DELORT-LAVAL, 1980).

Pour tester l'influence de la présence d'un produit formolé sur la fraction non tannée d'un aliment composé, un mélange de deux composants, farine de blé et tourteau de soja ou de colza tannés industriellement a été réalisé en faisant varier leurs proportions (farine de blé /tourteau : 0 /100; 25 /75; 50 /50; 100 /0). Une partie de ces mélanges a été agglomérée, sans addition d'eau, dans une presse expérimentale à piston (MELCION et DELORT-LAVAL, 1977) dont la température de filière était réglée à 80 °C. Enfin, pour simuler l'action de la vapeur dans une presse industrielle, une fraction des mêmes mélanges a été autoclavée pendant 1 mn à 115 °C, avant agglomération dans la même presse.

2.3. — *Analyses*

2.31. — *Formol*

Le titre des solutions concentrées de formol est vérifié par la méthode au sulfite de sodium (WALKER, 1964). Fe et Fh sont dosés suivant la méthode proposée dans une publication précédente (TOMÉ *et al.*, 1979).

2.32. — *Solubilité de l'azote*

La mesure de la solubilité est celle appliquée par VÉRITÉ, JOURNET et JARRIGE (1979) aux aliments du ruminant et non encore publiée. Deux grammes d'échantillon broyés et homogénéisés sont mis en suspension dans 50 ml de tampon de MAC DOUGALL (1948) ajusté à pH 6,9 par barbotage de gaz carbonique. Cette suspension est agitée pendant une heure à température ambiante puis centrifugée pendant 15 mn à 3 000 g. Le surnageant est recueilli dans une fiole de 100 ml. Le culot est repris dans 40 ml de la solution tampon, agité une heure et centrifugé comme précédemment. Le second surnageant est ajouté au précédent; la fiole est ajustée à 100 ml. L'azote est déterminé sur l'échantillon initial et sur le surnageant selon la méthode KJELDAHL. La solubilité azotée est exprimée en p. 100 de l'azote introduit.

2.33. — *Désamination en « rumen artificiel »*

Le matériel et les conditions des essais sont ceux décrits par TISSERAND et ZELTER (1965). L'inoculum est constitué par du liquide de panse filtré sur 6 couches de gaze. Les donneurs sont trois moutons mâles adultes castrés, nourris *ad libitum* avec du fourrage; ils sont mis à jeun 16 h avant le prélèvement. Dans chaque tube d'incubation sont introduits :

— une prise d'essai choisie de manière à apporter environ 60 mg d'azote;

— 20 ml d'inoculum;

— 20 ml de salive artificielle (MAC DOUGALL, 1948) enrichie en S et en oligo-éléments (KUMARESAN, 1976). En mg /l : Na_2SO_4 : 400 — NaCl : 470 — KCl : 450 — $MgCl_2$ anhydre : 47 — $CaCl_2$ anhydre : 55 — $NaHCO_3$: 9 240 — Na_2HPO_4, 12 H_2O : 7 125. Un litre de cette solution est supplémenté par 10 ml d'une solution d'oligoéléments ayant la composition suivante, en mg /l : $ZnSO_4$: 1 320; $CoCl_2$, 6 H_2O : 12; $MnSO_4$, H_2O : 1 890; Fe SO_4, 7 H_2O : 3 680; Na_2SeO_3, 5 H_2O : 2; $CuSO_4$, 5 H_2O : 216; $(NH_4)_6$ Mo_7 O_{24}, 4 H_2O : 8,7; Na I : 1,65.

Les essais sont répétés trois fois. Un tube témoin, contenant la même quantité de matière sèche sous forme de paille broyée est incubé dans les mêmes conditions en vue de la correction de l'essai (ZELTER et LEROY, 1966). Après 24 h d'incubation, 10 ml de solution d'acide trichloracétique à 10 p. 100 (p /v) sont ajoutés dans chaque tube. Les tubes sont centrifugés à 3 000 g pendant 5 mn, le culot est lavé à l'eau distillée et centrifugé à nouveau. Les surnageants provenant des deux centrifugations sont réunis et ajustés à 100 ml dans une fiole jaugée avec de l'eau distillée. L'ammoniac apparu est dosé sur un appareil automatique (CROOKE et SIMPSON, 1971). Le taux de désamination est exprimé par le rapport :

$$\frac{(N._{NH_3}\ \text{essai} - N._{NH_3}\ \text{témoin})}{N\ \text{prise d'essai}} \times 100$$

3. — Résultats

3.1. — *Influence du formol mis en jeu sur les caractéristiques des produits tannés suivant la méthode de référence*

Pour les différents aliments expérimentaux, lorsque la proportion de formol mise en jeu s'accroît, Fe et Fh augmentent également (fig. 1). La quantité de Fh dosée est toujours inférieure à la quantité de formol mise en jeu : au cours de la lyophylisation, une fraction de formol est perdue par évaporation et une autre se fixe de manière irréversible (Tome *et al.*, 1979). Les relations entre le formol mis en jeu et Fe ou Fh sont très sensiblement linéaires, dans les conditions de nos essais, les régressions linéaires étant, dans tous les cas, hautement significatives. La valeur des intersections à l'origine des droites de régression n'est pas significativement différente de 0. Une analyse de covariance entre Fh et Fe et le formol mis en jeu conduit à admettre ($P < 0{,}01$) que le formol ne se fixe pas avec la même intensité sur les différents substrats (tabl. 1).

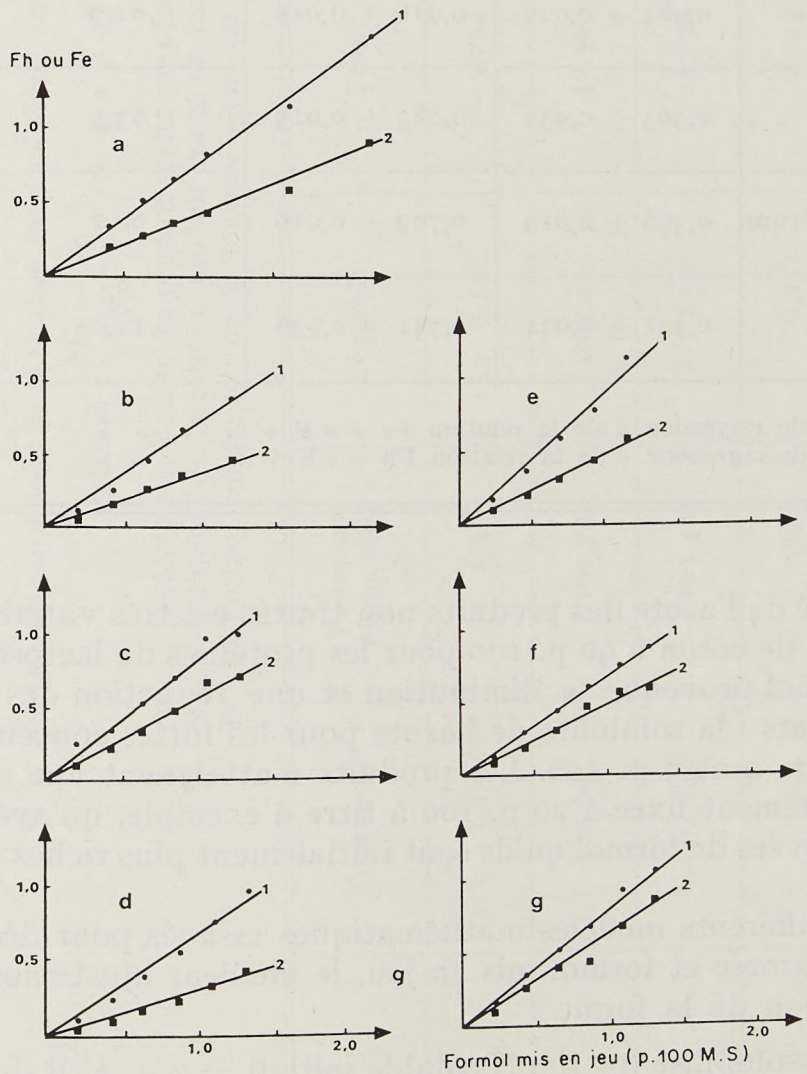

Fig. 1. — *Influence du taux de formol ajouté sur les teneurs en formol libérable par hydrolyse* (1) *et en formol extractible* (2) *de différents aliments traités par la méthode de référence : protéines de lactoserum* (a), *fèverole* (b), *tourteau d'arachide* (c), *tourteau de coton* (d), *pois* (e), *lupin* (f), *tourteau de tournesol* (g).

TABLEAU I

Influence de la quantite de formol mise en jeu (F) sur le formol extractible (Fe), le formol libéré par hydrolyse (Fh) et sur la solubilité azotée

	Fe/F (1)	Fh/F (2)	N soluble p. 100 total avant traitement	Quantité de formol (p. 100 MS) nécessaire pour réduire la solubilité azotée à 20 p. 100 du N total
Tourteau de tournesol .	0,616 ± 0,042	0,796 ± 0,017	30,5	0,13
Tourteau d'arachide. .	0,564 ± 0,034	0,808 ± 0,046	56,6	0,25
Fèverole.	0,401 ± 0,025	0,760 ± 0,052	73,5	0,55
Pois.	0,484 ± 0,020	0,916 ± 0,045	82,9	0,43
Lupin	0,503 ± 0,030	0,683 ± 0,015	83,3	1,01
Protéines de lactosérum	0,396 ± 0,019	0,729 ± 0,016	90,1	1,05
Tourteau de coton. . .	0,341 ± 0,024	0,741 ± 0,036	12,0	—

(1) Coefficient de régression a de la relation $Fe = a.F + b$.
(2) Coefficient de régression c de la relation $Fh = c.F + d$.

La solubilité de l'azote des produits non traités est très variable, de 12 p. 100 pour le tourteau de coton à 90 p. 100 pour les protéines de lactosérum. Le traitement par le formol provoque sa diminution et une réduction des écarts entre les différents substrats : la solubilité de l'azote pour les fortes concentrations en formol se situe entre 4 et 9 p. 100. Les produits n'atteignent une solubilité azotée donnée, arbitraitement fixée à 20 p. 100 à titre d'exemple, qu'avec des quantités d'autant plus élevées de formol qu'ils sont initialement plus riches en azote soluble (tabl. 1).

Parmi les différents modèles mathématiques essayés pour décrire la relation entre solubilité azotée et formol mis en jeu, le meilleur ajustement a été obtenu pour une équation de la forme :

$$\text{N insolubilisé (p. 100 N soluble initial)} = a + b\sqrt{F} + cF$$

où a, b, c, sont des coefficients dépendant de la nature des produits testés et F, la dose de formol mise en jeu exprimée en pourcentage (p/p) par rapport à la matière sèche du substrat (tabl. 2).

TABLEAU 2

Valeur des coefficients de la relation entre solubilité azotée et formol mis en jeu

$$\text{Relation : } \frac{No - Ne}{No} \cdot 100 = a + b\sqrt{F} + cF$$

avec : No = Solubilité de l'azote du produit traité sans formol (N soluble % N total)
Ne = Solubilité de l'azote du produit traité au formol (N soluble % N total).
F = Formol (en p. 100 de la matière sèche du substrat).

	Tournesol	Tourteau d'arachide	Fèverole	Pois	Lupin	Protéines de lactosérum	Tourteau de coton
a	1,439	1,58	— 3,555	— 5,004	— 2,448	— 3,585	— 0,743
b	175,66	196,755	107,405	47,56	132,898	39,474	85,865
c	— 92,249	— 105,405	— 10,592	28,676	— 46,758	23,274	— 31,073
r	0,991	0,994	0,952	0,944	0,979	0,950	0,994

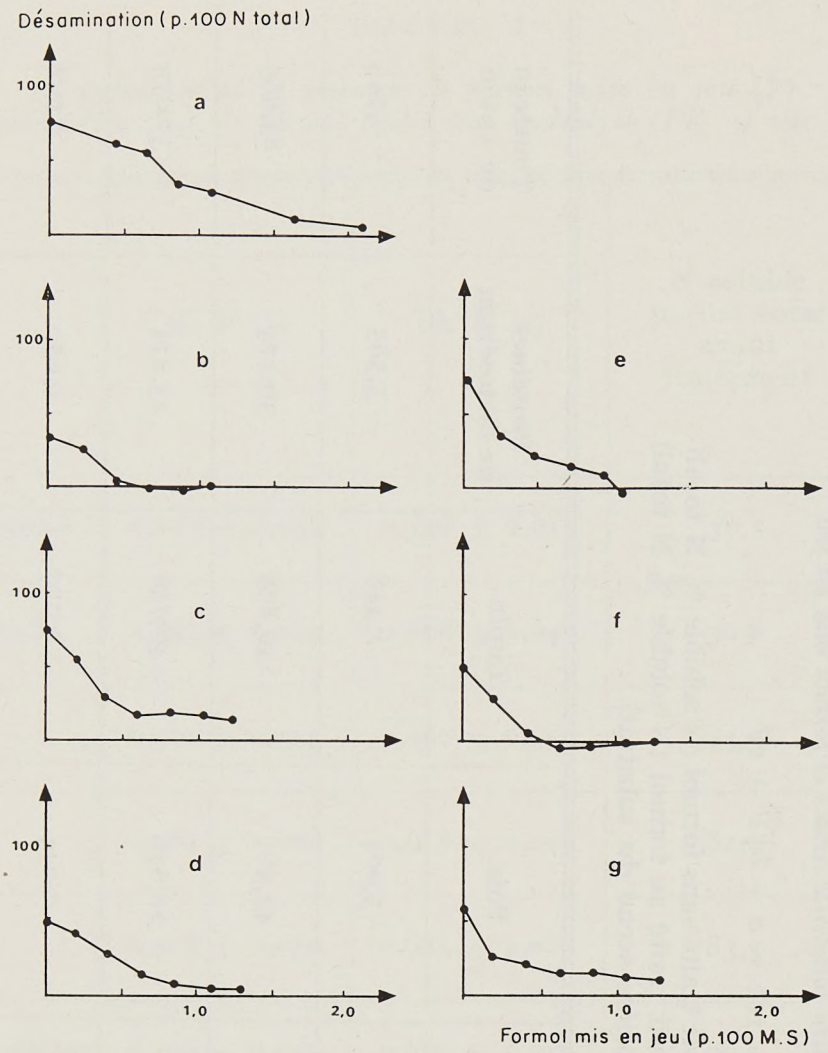

FIG. 2. — *Influence du taux de formol ajouté sur l'intensité de la desamination* in vitro *des protéines de différents aliments traités selon la méthode de référence : protéines de lactosérum* (a), *fèverole* (b), *tourteau d'arachide* (c), *tourteau de coton* (d), *pois* (e), *lupin* (f), *tourteau de tournesol* (g).

Dans les conditions du test de désamination *in vitro*, le traitement des aliments par le formol provoque une diminution de l'ammoniac apparu après 24 h (fig. 2). Le taux de désamination prend quelquefois des valeurs négatives, le tube témoin dosant plus d'ammoniac que l'essai à la fin de l'incubation. Ceci est vraisemblablement une conséquence de la différence d'apport énergétique entre l'essai et le témoin, ce qui influe sur la consommation d'ammoniac au cours de la fermentation. Pour tenir compte de l'énergie disponible au cours du test *in vitro*, un autre mode d'expression est proposé dans la partie « DISCUSSION ».

3.2. — *Effet du mode de traitement par le formol*

Le pois a été traité à la fois par la méthode de référence et par pulvérisation de formol concentré. Pour des quantités comparables de formol mises en jeu, la concentration en formol est entre 40 et 200 fois plus importante dans la solution pulvérisée que dans celle de la méthode de référence (fig. 3 et 4).

A dose de formol mise en jeu comparable, l'augmentation de sa concentration provoque un accroissement léger du formol réversiblement lié (Fr), une diminution de la solubilité de l'azote et de la désamination *in vitro* (fig. 3 et 4) : ainsi, le traitement apparaît globalement plus intense lorsque la quantité d'eau ajoutée est plus faible.

A la température ambiante et dans le cas du pois traité par 0,8 p. 100 (p /p) de formaldehyde pur suivant la méthode de référence, la plus grande partie des réactions entre le formol et l'aliment semble se produire sur une durée inférieure à 24 h. La solubilité azotée, initialement voisine de 80 p. 100 s'abaisse à 20 p. 100 environ en 24 h, puis reste pratiquement inchangée au-delà. Par contre, Fr augmente régulièrement et faiblement pendant les huit premiers jours et se stabilise ensuite.

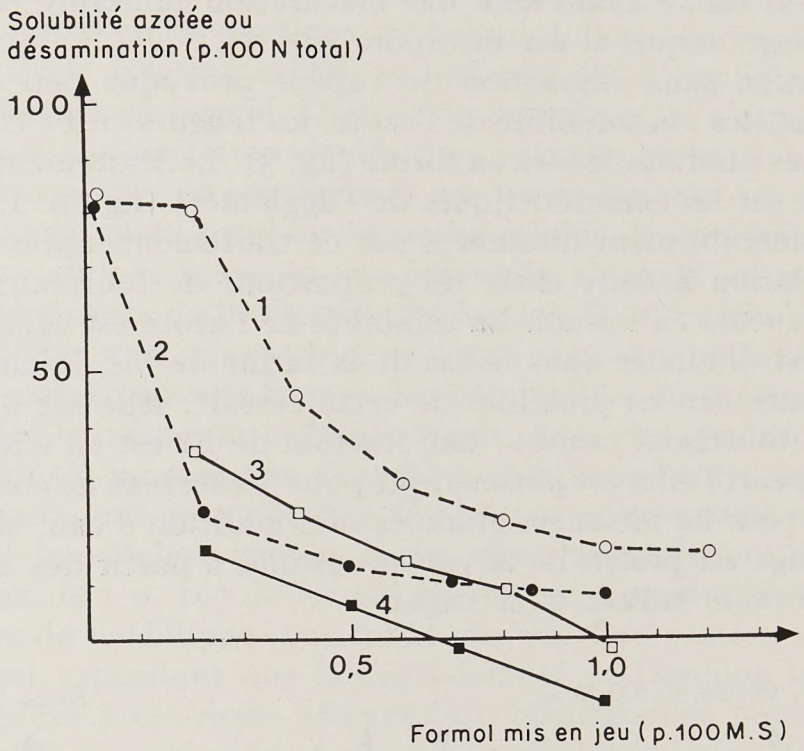

FIG. 3. — *Effet de la concentration de la solution de formol sur la solubilité de la fraction azotée et sur la désamination* in vitro *du pois : solubilité azotée après traitement par des solutions diluées* (1) *ou concentrées* (2); *désamination après traitement par des solutions diluées* (3) *ou concentrées* (4).

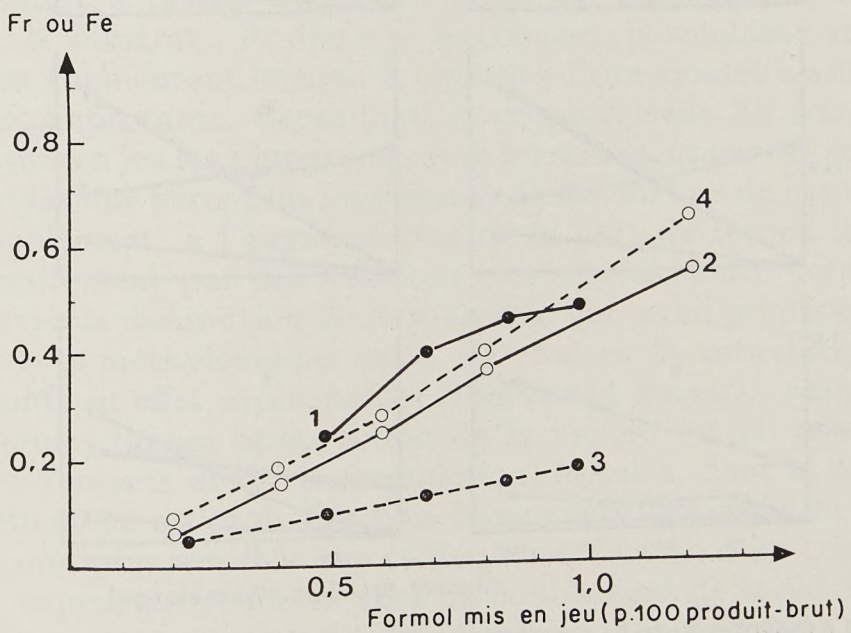

FIG. 4. — *Effet de la concentration de la solution de formol sur la teneur en formol extractible* (*Fe*) *et en formol réversiblement lié* (*Fr*) *du pois : Fr en solution concentrée* (1) *ou diluée* (2); *Fe en solution concentrée* (3) *ou diluée* (4).

3.3. — *Effet des traitements technologiques après tannage*

Dans les mélanges modèles de farine de blé et de tourteau de colza ou de soja tanné, les quantités de formol retrouvé sous ses différentes formes sont très proches de celles apportées par le substrat formolé (fig. 5). La quantité d'azote soluble du mélange est également assez voisine de la somme de celles apportées par chacun des constituants. Il n'y a donc pas de formation de liaisons nouvelles entre le formol d'un substrat tanné ayant subi une maturation suffisante et la fraction non tannée du mélange auquel il est incorporé (fig. 5).

La granulation sans adjonction de vapeur provoque peu de modifications des mélanges modèles : la solubilité de l'azote, les teneurs en Fe et Fh sont comparables à celles des produits laissés en farine (fig. 5). Le traitement par la vapeur a un effet marqué sur les caractéristiques de l'aggloméré (fig. 5). Les teneurs en Fe et Fh sont considérablement diminuées par ce traitement, après lequel il ne subsiste plus de relation linéaire entre les proportions de tourteaux tannés dans le mélange et les teneurs en formol. La solubilité de l'azote est également fortement modifiée : elle est diminuée dans le cas de la farine de blé, la chaleur ayant une action dénaturante sur les protéines de cette céréale. Elle est au contraire augmentée avec les tourteaux tannés : une fraction de Fr est en effet entraînée sous l'action de la vapeur; l'effet est plus marqué pour le tourteau de colza que pour celui de soja. Comme pour les mélanges granulés sans addition d'eau, la teneur en azote soluble du mélange est proche de la valeur calculée à partir des analyses séparées des deux composants traités à la vapeur.

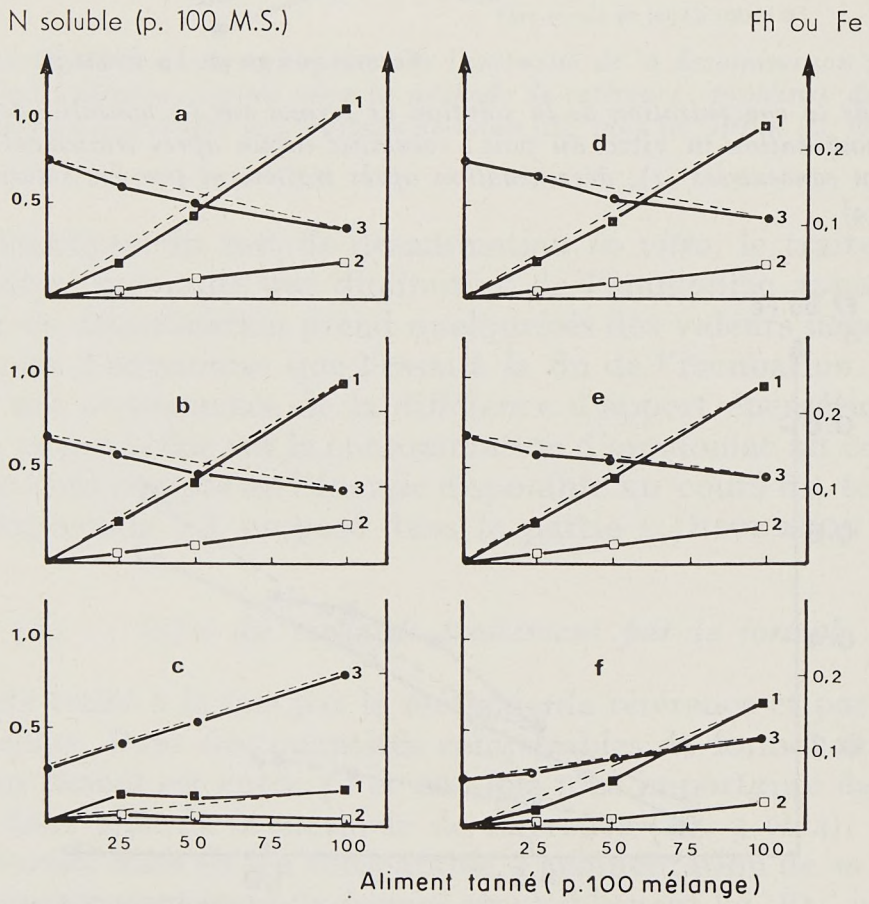

FIG. 5. — *Effet de l'incorporation de tourteaux tannés à de la farine de blé sur la teneur en formol libérable par hydrolyse* (1) *ou extractible* (2) *et en azote soluble* (3). *Les valeurs calculées sont indiquées en traits pointillés. Colza ou soja : en farine* (a *ou* d), *aggloméré* (*b ou* e), *autoclavé et aggloméré* (c *ou* f).

4. — Discussion

4.1. — *Relation entre les paramètres des traitements et les propriétés des aliments*

Les relations linéaires observées entre le taux de formol mis en jeu et Fe ou Fh montrent que les sites réactifs susceptibles de fixer le formol par des liaisons de faible énergie ou par des liaisons covalentes sont loin d'être complètement saturés, même aux plus fortes concentrations en formol du présent essai. Cependant, les caractéristiques de la fraction azotée ne suffisent pas à expliquer les différences observées de rétention en formol. Dans cette expérience, il n'a pas été possible de mettre en évidence de corrélation significative entre les proportions de Fe ou de Fh par rapport au formol mis en jeu (estimée par les coefficients des droites de régression) et la teneur en azote total ou en azote soluble des produits. De même, les teneurs en acides aminés basiques des différents aliments ne semblent pas être les facteurs déterminants de la fixation du formol. Il est connu que de nombreux composés biologiques sont susceptibles de réagir avec cette substance : les groupes hydroxylés des sucres peuvent former des hémiacétals en présence de l'aldéhyde (Walker, 1964). De même, la cellulose réagit, dans des conditions douces : une étude de Roff (1958) montre que l'équilibre réactionnel entre formol et cellulose demande environ un jour pour s'établir; la réaction est réversible sous l'action d'un lavage à l'eau de la cellulose traitée. Enfin, dans les conditions du traitement de référence, au taux de 1 p. 100 de formol mis en jeu, l'amidon de maïs purifié fixe environ 50 p. 100 de l'aldéhyde sous forme de Fh. Des essais *in vitro* en rumen artificiel montrent cependant que la dégradabilité de l'amidon n'est pas affectée par le formol ajouté à ces doses (Bertrand, résultats non publiés). La présence d'eau ou de réactifs accepteurs de formol, tels que l'ammoniac, déplace l'équilibre en diminuant la quantité de formol fixé sur l'amidon. Cette réaction pourrait intervenir dans le rumen, en présence de l'ammoniac provenant de la désamination des protéines alimentaires.

La comparaison des résultats obtenus par les deux modes de tannage au laboratoire conduit à penser que l'eau aurait un effet inhibiteur des réactions du formol avec le substrat : Fr diminue légèrement, la solubilité azotée et le taux de désamination augmentent lorsque la quantité d'eau ajoutée au moment du traitement est plus importante. Cependant, la proportion de Fh retrouvée par rapport au formol mis en jeu est plus grande avec le traitement par des solutions diluées. Ceci peut être dû à une perte plus importante de formol lors de la pulvérisation ou, plus vraisemblablement, à l'augmentation de la part du formol irréversiblement lié durant le traitement par des solutions concentrées. Cette dernière hypothèse est en accord avec la diminution de la solubilité qui serait principalement due à la formation de ponts méthyléniques entre les chaînes de protéines : certaines de ces réactions sont, en effet, irréversibles (French et Edsall, 1945). La solubilité azotée des produits tannés après action de la vapeur est en relation avec celle mesurée sur les aliments avant la formolation; le colza, dont la solubilité azotée avant tout traitement est trois fois plus importante que celle du soja est, après tannage, beaucoup plus sensible que ce dernier à l'action d'un traitement hydrothermique. La vapeur provoque en effet un doublement de la solubilité azotée du colza tanné et une augmentation de seulement 7 p. 100 en valeur absolue pour le soja formolé. Ces résultats montrent la réversibilité partielle des modifications engendrées par le tannage. Dans un autre essai portant sur un mélange contenant

des substrats traités par le formol et agglomérés industriellement avec injection de vapeur, il a été observé (TISSIER, communication personnelle) une augmentation de la solubilité azotée de 9 p. 100 et de la désamination de 7 p. 100 en valeur absolue par rapport au témoin non aggloméré. L'effet de l'injection de vapeur lors de l'agglomération industrielle est donc suffisamment sensible pour provoquer une modification de la valeur azotée d'un aliment composé préalablement traité par le formol.

La présente étude de l'influence de l'eau, liquide ou à l'état de vapeur, sur l'efficacité du tannage fait apparaître l'existence de deux types de liaisons responsables de l'insolubilisation des protéines; les unes seraient réversibles, les autres irréversibles et leur importance relative reste à déterminer. Après hydrolyse acide de protéines traitées par le formol, on retrouve, comme ponts de réticulation, des liaisons de type « MANICH » entre lysine et tyrosine, de nature irréversible (FRAENKEL-CONRAT et OLCOTT, 1948*b*; BLASS, BIZZINI et RAYNAUD, 1967). D'autre part, de nombreuses liaisons peuvent redonner du formol libre dans des conditions relativement douces (TOME *et al.*, 1979). Parmi celles-ci, certaines ne sont vraisemblablement pas liées à l'insolubilisation des protéines; c'est le cas en particulier des dérivés hydroxyméthylés (FRENCH et EDSALL, 1945).

Au contraire, d'autres liaisons de réticulation, faisant intervenir notamment les groupements amine et amide primaires, guanidyle, thiole et peut-être indole et imidazole (FRAENKEL-CONRAT et OLCOTT, 1948, *a* et *b*) qui pourraient être également réversibles participeraient à l'insolubilisation des composés azotés. Le traitement par la vapeur étudié agirait sur ce dernier type de liaison que permet d'évaluer Fr (TOME *et al.*, 1979).

4.2. — *Relation entre solubilité azotée et désamination*

De nombreuses études ont montré l'existence d'une relation entre solubilité de l'azote et désamination « *in vitro* » (JARRIGE, 1978; CRAWFORD *et al.*, 1978; THOMAS, TRENKLE et BURROUGHS, 1979). Dans nos conditions expérimentales, on observe effectivement la relation linéaire suivante :

$$DES_a = 0{,}58 . SOLN + 11 \qquad (n = 38;\ r = 0{,}73)$$

avec :

DES_a : taux de désamination apparent après 24 h d'incubation *in vitro* (en pourcentage d'N total)).

SOLN : solubilité azotée (N soluble p. 100 N total)).

En appliquant un autre mode d'expression de la désamination dans lequel la teneur en azote ammoniacal de l'essai est corrigée de la valeur initiale au début de l'incubation :

$$DES_b = \frac{N._{NH_3}\ \text{essai} - N._{NH_3}\ \text{initial}}{N\ \text{prise d'essai}} . 100$$

on obtient la régression suivante :

$$DES_b = 0{,}68 . SOLN - 1{,}7 \qquad r = 0{,}77$$

Les coefficients de ces régressions étant assez faibles, il a paru intéressant de rechercher une nouvelle expression de la désamination tenant compte de la valeur énergétique de l'aliment : en effet, la synthèse d'azote microbien pendant la durée

du test correspond à une disparition d'ammoniac et conduit à une sous-estimation de la désamination, d'autant plus importante que la quantité d'énergie disponible apportée par l'aliment est élevée. Pour établir un mode de correction, il a été admis que :

— l'azote ammoniacal disparu sous l'action des microorganismes est proportionnel à la quantité de matière organique digestible (MOD) apportée dans l'essai; cette quantité est évaluée à partir de tables de valeurs nutritives (JARRIGE, 1978);

— le liquide de rumen n'apporte qu'une quantité négligeable d'énergie fermentescible; l'azote insoluble du liquide de rumen au début de l'essai ne donne pas naissance à de l'azote ammoniacal.

Ces hypothèses sont évidemment critiquables :

— la liaison entre énergie fermentescible et MOD est assez lâche et peut être variable en fonction de la nature des aliments, comme le laissent supposer les résultats publiés par JARRIGE (1978).

— une partie des microorganismes se lysant au cours de l'incubation, le liquide de rumen peut apporter une fraction non négligeable de l'azote soluble présent à la fin de l'essai.

Pour éviter d'exploiter des données de désamination provenant d'essais dans lesquels l'azote fermentescible est le facteur limitant de la fermentation, nous n'avons tenu compte que des résultats provenant d'expériences dans lesquelles la concentration finale en ammoniac dans le tube à essai était supérieure à 200 mg d'azote ammoniacal par litre.

Sur la base de ces hypothèses, la relation prend la forme :

$$DES_c = \frac{NF - NL + 0{,}16\ k.MOD}{NA} \cdot 100$$

avec DES_c = désamination corrigée (N désaminé p. 100 N total);
NA = azote apporté par l'aliment (mg);
NL = azote ammoniacal apporté par le liquide de rumen au début de l'essai (mg);
NF = azote ammoniacal présent à la fin de l'essai (mg);
MOD = matière organique digestible apportée par l'aliment (mg);
k = quantité de matières azotées microbiennes (N × 6,25) synthétisées (g/g MOD).

Les coefficients de corrélations obtenus entre la désamination corrigée et la solubilité azotée étant très voisins pour une large plage de valeurs de k, la relation la plus vraisemblable peut être déterminée en faisant l'hypothèse que la totalité de l'azote soluble est désaminée; c'est-à-dire que, pour un produit ayant une solubilité azotée de 100 p. 100, la désamination corrigée calculée est également de 100 p. 100. Cet ajustement est réalisé pour une valeur de k égale à 0,131 g de protéines brutes par gramme de MOD, valeur proche de celle (0,135) retenue par JARRIGE (1978), à partir d'essais *in vivo*. La relation entre désamination corrigée et solubilité azotée devient alors :

$$DES_c = 0{,}79.SOLN + 21 \qquad r = 0{,}88$$

La corrélation entre désamination et solubilité azotée est améliorée lorsqu'on tient compte de l'énergie apportée par l'aliment. De plus, cette équation de régression est en parfait accord avec les essais de VÉRITÉ et JOURNET (1979) sur vaches laitières, à partir d'un mélange de colza et de soja tannés : dans leur étude, la quantité d'azote non dégradé dans le rumen est estimée à 75 p. 100 de l'azote des tourteaux tannés, dont le taux de solubilité azotée est d'environ 5 p. 100.

5. — Conclusion

Les résultats des tests que nous avons appliqués à plusieurs sources de protéines alimentaires du ruminant (solubilité azotée, désamination, dosages du formol) précisent leurs caractéristiques physicochimiques et décrivent la répartition du formol dans les produits traités. Pour l'ensemble des substrats de ces essais, l'addition de doses croissantes de formol a pour effets une augmentation de Fe et Fh, une diminution de la solubilité azotée jusqu'à une valeur limite correspondant à l'azote non protéique, une réduction de la désamination *in vitro*. Seulement 20 p. 100 environ de l'azote insoluble d'un aliment tanné sont dégradés dans le rumen *in vitro*; ce taux est de 35 p. 100 pour un aliment non traité, valeur retenue pour l'estimation des Protéines Digestibles dans l'Intestin des aliments (JARRIGE, 1978).

TABLEAU 3

Quantités de formol nécessaires pour traiter différents aliments protéiques du ruminant

Substrat	Formol ajouté	
	En p. 100 de la matière sèche	En p. 100 des protéines brutes
Tourteau de Tournesol	0,5	1,2
Tourteau d'Arachide	0,7	1,3
Fèverole	0,45	1,5
Pois	0,9	3,4
Lupin	0,5	1,6
Protéines de lactosérum	1,25	1,7
Tourteau de coton	0,7	1,6

Les taux de formol à appliquer, d'après nos essais, pour un traitement correct des aliments (tabl. 3) sont différents et ne dépendent pas principalement de la teneur en azote des substrats. L'absence de relation significative entre la rétention du formol et le taux protéique ou la solubilité de la fraction azotée, laisse supposer que l'aldéhyde est susceptible de réagir également avec des composés non azotés de la ration. Dans ces conditions, il paraît plus logique de définir un traitement par la quantité de formol mise en jeu en pourcentage du produit, plutôt que par rapport aux protéines brutes ou solubles du substrat. Enfin, après maturation, les aliments traités semblent assez stables. Il est donc possible, en mélangeant un substrat formolé avec un produit non traité, de réaliser pratiquement une gamme d'aliments apportant des proportions différentes d'azote soluble et de s'adapter à diverses complémentations de la ration de base du ruminant.

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Summary

Formaldehyde treatment of dietary proteins for ruminants

II. — *Effect of formaldehyde level and of other treatments on nitrogen solubility and* in vitro *deamination*

7 protein-rich feeds (pea, fababean and lupine seeds, groundnut meal, sunflower meal, cotton seed meal, whey protein concentrate) were treated with increasing amounts of formaldehyde (F). Extractible formaldehyde (Fe) or formaldehyde released after hydrolysis (Fh), nitrogen solubility and « *in vitro* » deamination were determined. Linear relationships are obtained between the amount of added formaldehyde (F) and Fe or Fh. Regression cœfficients are significantly different between feeds ($P < 0.01$). Ratios Fh/F range from 0.68 (lupin) to 0.92 (pea). However, no obvious relationships are found between protein level or solubility and Fe or Fh. Nitrogen solubility is closely related with *in vitro* deamination ($r = 0,88$), when taking into account estimated organic matter digestibility and its effect on ammonia consumption by the microorganisms *in vitro*. Only 20 p. 100 of insoluble nitrogen in the treated product are apparently degraded in the rumen. By steam treatment before pelleting, a release of formaldehyde and an increased nitrogen solubility are observed. In presence of formaldehyde-treated rapeseed or soyabean meal, untreated feeds are not noticeably modified.

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Dosages fluorimétriques et densitométriques du coumestrol Application au contrôle de la qualité des préparations protéïques de feuille de luzerne (*Medicago sativa*)

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Résumé

Le coumestrol (7,12-dihydroxybenzofuranocoumarine) présente des spectres d'absorption et de fluorescence caractéristiques entre 250 et 450 nm (fig. 1 et 2). Après séparation chromatographique et élution des taches, les réponses fluorimétriques du coumestrol en solution méthanolique sont linéaires, au moins entre 0 et 250 ng par tache (fig. 3). Le coumestrol et les coumestanes présents dans les préparations protéiques de feuilles de luzerne peuvent être dosés également soit après extraction progressive sur colonne et fluorimétrie en solution soit après partage entre solvants et densitométrie sur chromatogramme (tabl. 1). Les coumestranes présents dans les préparations protéiques thermoprécipitées donnent lieu à compartimentation entre l'éther éthylique et le méthanol (tabl. 2); ils peuvent être complètement solubilisés par extraction au méthanol. Le lavage de préparations protéiques, thermoprécipitées ou polyprotéines, à l'aide de solvants organiques réduit significativement les teneurs des coumestanes (tabl. 3) mais aussi, dans certains cas, des flavonoïdes (fig. 4). L'origine du phénomène de compartimentation de ces polyphénols est discutée.

Mots-clés : *isolat. protéique, concentrat protéique, coumestrol, coumestanes, luzerne, densitométrie, fluorimétrie, compartimentation.*

Introduction

La présence de polyphénols non tanins, flavonoïdes et coumestanes, a été rapportée précédemment dans des préparations protéiques isolées à partir des broyats de parties aériennes de Luzerne (MONTIES et RAMBOURG, 1978*a*, *b*). Parmi les coumestanes, c'est le coumestrol (7,12-dihydroxybenzofuranocoumarine ou 7,12-coumestane) qui est en général le plus important pondéralement.

(**) A qui toute correspondance doit être adressée.

Les coumestanes, et à un degré moindre certains flavonoïdes, dont les structures moléculaires s'apparentent à celles de l'œstradiol peuvent exercer des effets œstrogènes néfastes sur la croissance et le reproduction des mammifères. De tels effets ont été rapportés récemment, par exemple, dans le cas de bouvillons et de génisses recevant des fourrages contenant du coumestrol; un seuil de 37 ppm a même été déterminé (Lookhart, 1980). Il semble cependant prudent de fixer la limite de risque d'effets antinutritionnels aux environs d'une dizaine de parties par million à cause de la diversité des effets biologiques des polyphénols et de la difficulté d'analyser la variabilité de leurs effets métaboliques (Monties, 1981).

Dans les préparations protéines obtenues tant à l'échelle pilote, par thermoprécipitation, qu'en laboratoire, par précipitation au moyen de solvants organiques, les teneurs en coumestanes sont de l'ordre de quelques parties par million alors que les teneurs en polyphénols totaux, estimées globalement après spectrophotométrie, sont de l'ordre de quelques pour cent de la matière sèche. Or, dans les parties aériennes de Luzerne, les quantités correspondantes de polyphénols sont fréquemment au moins dix fois supérieures. De plus, ces concentrations peuvent varier en fonction de nombreux facteurs (stade de développement, conditions d'environnement, type de cultivar, état sanitaire). On peut donc s'attendre à des variations corrélatives des teneurs des préparations protéiques correspondantes.

Il est donc important de pouvoir contrôler les variations de teneurs en coumestanes, même si leurs concentrations sont faibles en valeur absolue.

Dans ce travail nous avons, d'une part, mis au point une méthode simplifiée de dosage du coumestrol par densitométrie après extraction des polyphénols sur colonne, d'autre part nous avons contrôlé l'influence du lavage des préparations protéiques à l'aide de solvants organiques sur leur teneur en coumestanes et flavonoïdes.

Dans les deux cas les polyphénols ont été extraits par percolation sur colonne au moyen de solvants de polarités croissantes. Cette technique permet l'analyse du phénomène de compartimentation qui a été défini par le fait qu'un même polyphénol peut être extrait de façon exhaustive au moyen d'au moins deux de ses solvants employés successivement (Monties, 1975). Le phénomène de compartimentation semble résulter d'interactions non covalentes avec des structures définies insolubles (Monties, 1975; Monties et Rambourg, 1978 *a*; Monties, 1981). Récemment Andersen *et al.* (1980) ont cependant proposé d'expliquer la compartimentation des alcools hydroxycinnamiques dans les tiges de Luzerne par des inter-actions covalentes. Dans ce travail, nous avons plus particulièrement étudié la compartimentation du coumestrol rapportée par ailleurs (Monties et Rambourg, 1978 *a*).

Techniques expérimentales et matériel

A. — *Isolement des préparations protéiques*

Sauf indication contraire, les isolats protéiques ont été préparés en atelier pilote (France Luzerne) par la méthode dite de trempage. Les luzernes broyées sont mises à tremper dans un bain d'eau à une température de 70 ± 5 °C. Après pressage, un coagulum protéique vert est séparé du jus de presse (jus brun) qui contient en solution les protéines dites blanches. Ces protéines sont thermopré-

cipitées par injection de vapeur dans le jus brun. La pâte protéique ainsi précipitée est ensuite séchée par lyophilisation ou bien lavée par de l'éthanol à 95° puis de l'eau avant séchage. Ces préparations protéiques, « isolats », sont ensuite stockées à — 20° environ (GASTINEAU et de MATHAN 1981).

L'échantillon de protéine précipitée à l'aide de polyélectrolytes, « polyprotéines », a été fourni par le Dr R. FIORENTINI — Instituto di industrie agrarie — Pise — Italie. Préparée à partir de luzernes locales, la préparation protéique a été précipitée par addition d'un copolymère cationique PRODEFLOC C/1 (ANELLI *et al.*, 1977) puis décolorée par extraction dans un appareil de SOXHLET par l'acétone.

B. — *Extraction des polyphénols solubles*

Pour permettre l'étude de la compartimentation des polyphénols, l'extraction est faite au moyen d'une série de solvants de polarité croissante : pentane contenant éventuellement 2 p. 100 de méthanol, éther éthylique, méthanol, eau. La percolation a lieu dans une colonne sur 500 mg de protéine dispersés dans du sable selon un protocole décrit par ailleurs (MONTIES, 1975). Les fractions sont analysées par spectrophotométrie entre 240 et 750 nm; les teneurs en flavonoïdes sont exprimées en équivalent de quercétine (PM 300; $\varepsilon_{370} = 20\,000$).

C. — *Dosage du coumestrol*

Le coumestrol est dosé par référence à des gammes d'étalonnage préparées au moyen d'un échantillon fourni par le Dr BICKOFF E. M. (Western Regional Research Center, U.S.D.A. Berkeley, U.S.A.). Les solutions de référence dans le méthanol sont préparées par dilution au dixième d'une solution mère, à saturation, dans le méthanol. La concentration en coumestrol de la référence est calculée en fonction de son absorbance (PM = 268; $\varepsilon_{343} = 22\,000$). L'échantillon cristallisé n'a pas été repurifié.

1. — *Séparation par chromatographie sur couche de cellulose*

Les fractions méthanol (MeOH) et éther éthylique (EtOEt) obtenues par extraction progressive sont concentrées à 100 ml. Des aliquotes correspondant à 400 mg sont concentrées à environ 5 ml à l'aide d'un évaporateur rotatif, transférées dans un tube à vis, puis concentrées sous jet d'azote et reprises exactement dans 1 ml (extrait EtOEt) et 5 ml (extrait MeOH). La séparation chromatographique est faite sur plaque de cellulose MERCK « prêtes pour l'emploi ». A l'aide d'une seringue de 25 µl munie d'un « repeating dispenser » Hamilton, on dépose des charges de 20 µl, à 1,5 cm du bas de la plaque, sous forme de traits de 0,8 cm espacés de 1 cm. En plus des charges précédentes la même plaque porte une gamme de référence de coumestrol formée par des dépots de 5, 10, 15, 20 et 25 µl d'une solution de référence à 10 ng/µl de coumestrol dans le méthanol. Après développement à température ambiante et à l'obscurité par le mélange AHW (acide acétique + acide chlorhydrique + eau : 60 + 6 + 30 en volume), la plaque est séchée rapidement dans une étuve ventilée à 110 °C.

2. *Densitométrie des taches sur couche mince*

Le dosage est réalisé à l'aide d'un densitomètre VITRATON TLD 100 par fluorimétrie (mode lin + II) avec comme source une lampe à vapeur de mercure munie d'un filtre à large bande (filtre UV.B). La détection est faite à 452 nm ou à 515 nm selon que la charge moyenne à détecter est inférieure ou supérieure à 50 ng par tache. Les filtres d'analyse ont une bande passante de 10 nm environ.

3. *Fluorimétrie après extraction des taches*

Les taches de coumestrol, repérées par fluorimétrie sous filtre UV.B, sont grattées, transférées sur filtre en verre fritté n° 4. Le coumestrol est élué par 7 à 8 ml de méthanol. L'extrait, ajusté à 10 ml, est ensuite analysé par fluorimétrie.

La fluorescence est mesurée par excitation à 362 nm et détection à 415 nm à l'aide d'un spectrofluorimètre JOBIN et YVON JY 3 équipé d'un voltmètre numérique. Le zéro de lecture est donné par le méthanol pur (PROLABO RP) alors que le 100 p. 100 est ajusté sur la réponse correspondant au coumestrol extrait de la tâche 25 µl de la gamme. La courbe étalon de variation du pourcentage de fluorescence est ensuite construite au moyen des réponses obtenues avec les extraits des autres charges de la gamme.

D. — *Caractérisation des flavonoides*

Les flavonoïdes ont été identifiés de façon préliminaire grâce :

1 — à leurs spectres ultraviolets : présence de deux maximums de hauteur voisins centrés aux environs de 270 et 325 nm; ce dernier étant déplacé à 375 nm en présence de méthylate de sodium;

2 — leur Rf sur couche de cellulose dans le mélange AHW : tâches bien résolues de Rf 0,24 — 0,39 et 0,54 et trainée diffuse entre Rf 0,73 et 0,92;

3 — les réactions colorées caractéristiques de flavones de ces tâches : couleur brune sous UV.B fluorescent en jaune sous vapeur d'ammoniaque, et donnant des colorations jaune vif à jaune orangé avec le réactif de neu (MONTIES et RAMBOURG, 1978*a*).

Résultats

La figure 1 présente le spectre d'absorption dans l'ultraviolet proche du coumestrol qui possède le profil d'absorption caractéristique des coumestanes, avec une zone de minimums mal définis vers 300 nm et un maximum à 343 nm dans le méthanol.

Dans la figure 2 sont représentés les spectres d'excitation et d'émission de la fluorescence du coumestrol dans le méthanol. Les mesures ont été faites respectivement à 415 et 362 nm; ces maximums ont été déterminés par tatonnement en début d'analyse.

La figure 3 présente les points expérimentaux correspondant au dosage étalon du coumestrol par fluorimétrie après élution des tâches séparées sur deux plaques différentes. Les résultats sont reproductibles et la courbe de réponse

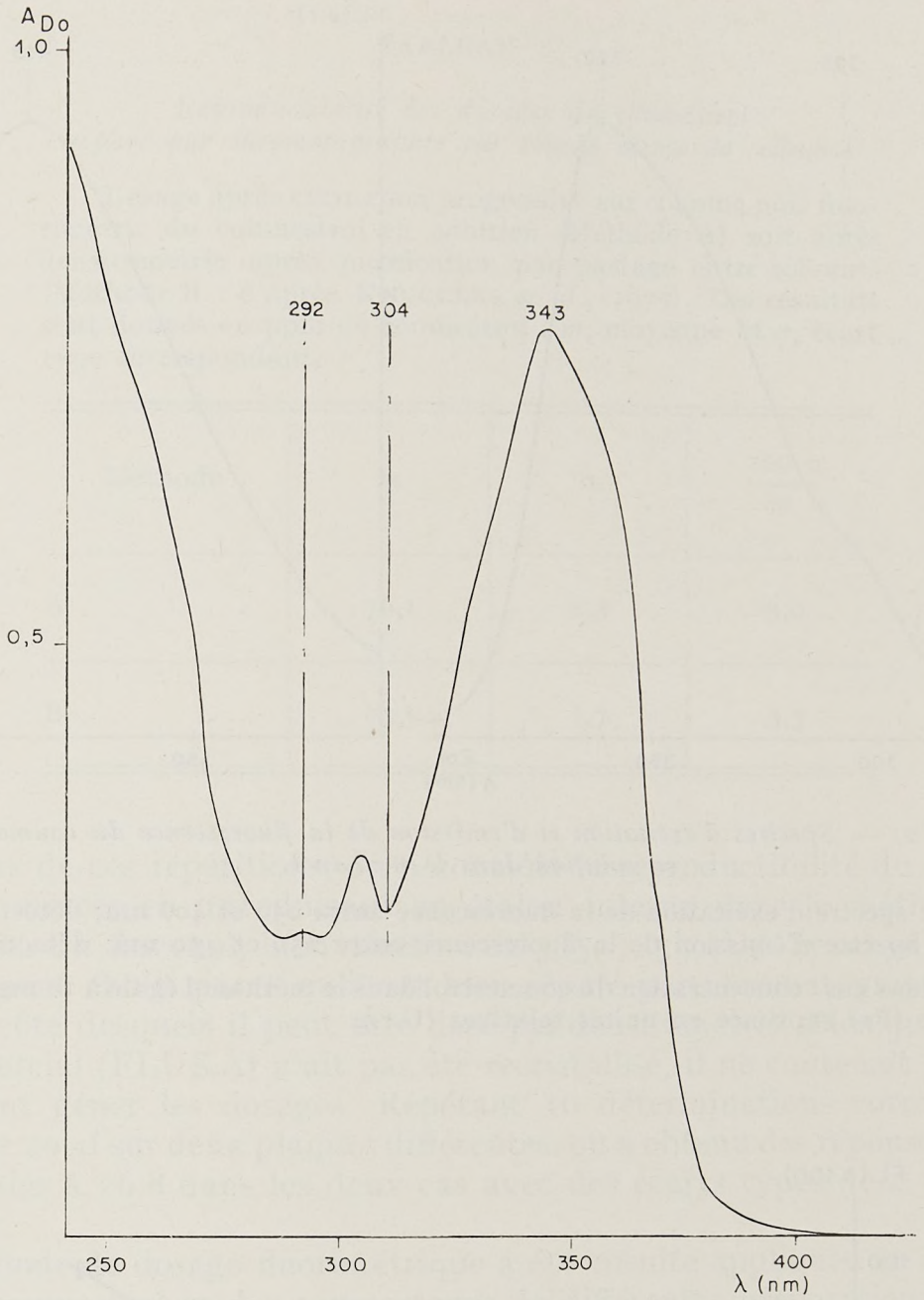

FIG. 1. — *Spectre d'absorption du coumestrol en solution dans le méthanol.*

est linéaire entre 0 et 250 ng par tâche. Des résultats semblables ont été obtenus pour des gammes analogues comprises entre 5 et 50 ng ou 25 et 250 ng en pratiquant les lectures fluorimétriques respectivement à 452 et 515 nm.

Les résultats des dosages du coumestrol par densitométrie sur couche mince et par fluorimétrie en solution ne différant pas de façon significative, nous avons comparé la méthode fluorimétrique à la méthode par densitométrie sur chromatogramme proposée par KNUCKLES *et al.* (1975). Selon ces derniers, le coumestrol, extrait par macération des produits dans des solvants organiques est séparé par partage entre solvants puis il est dosé par densitométrie après purification par chromatographie sur papier. Le protocole préconisé par KNUCKLES a donc été suivi sauf pour la chromatographie qui a été faite ici sur couche mince de cellulose.

Dans le tableau 1 sont comparés les résultats de plusieurs répétitions d'analyse faites sur un même échantillon de préparation protéique de Luzerne. On ne constate pas de différence significative entre les deux méthodes.

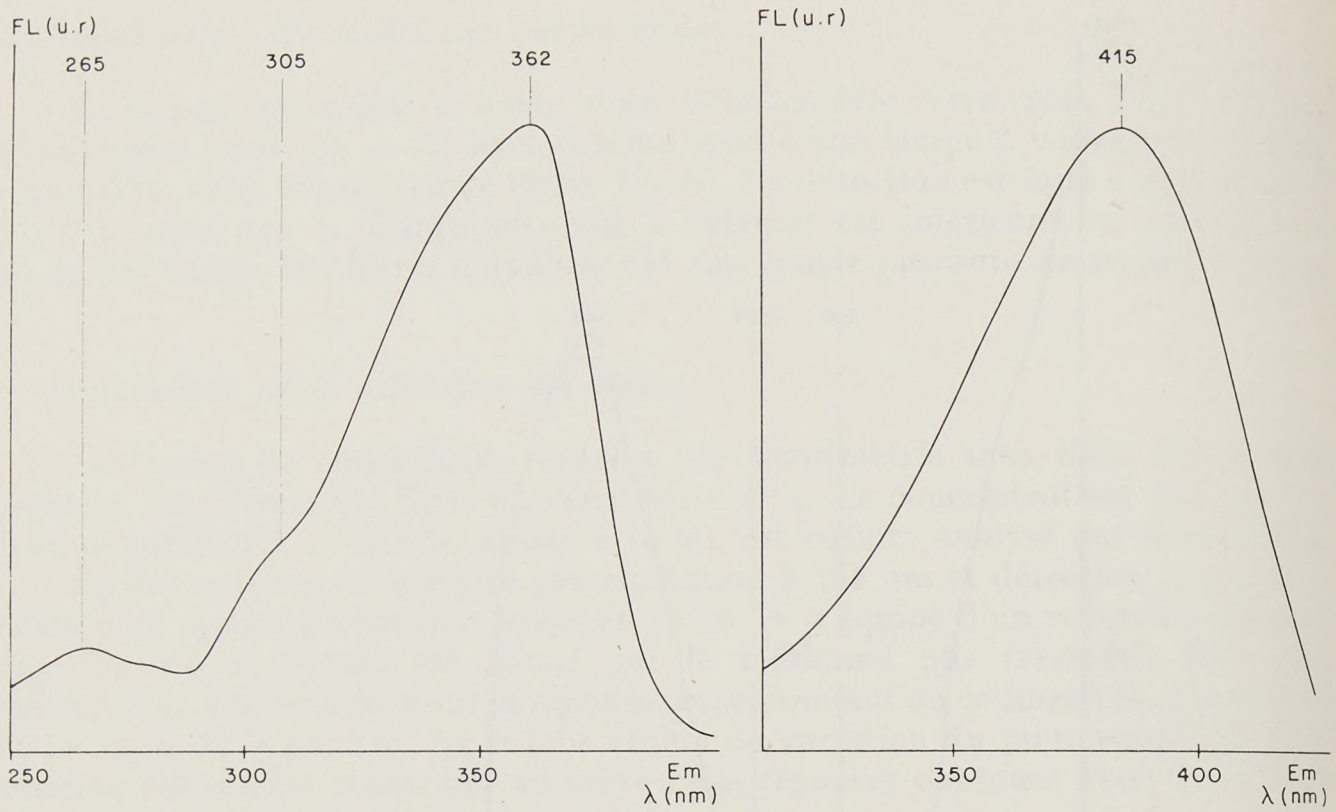

FIG. 2. — *Spectres d'excitation et d'émission de la fluorescence du coumestrol en solution dans le méthanol.*

A gauche : spectre d'excitation de la fluorescence entre 250 et 400 nm; détection à 362 nm.
A droite : spectre d'émission de la fluorescence entre 250 et 450 nm; détection à 415 nm.

Dans les deux cas : concentration du coumestrol dans le méthanol égale à 10 mg /l ($3.7\ 10^{-5}$M) et fluorescence (F1) exprimée en unités relatives (U.r.).

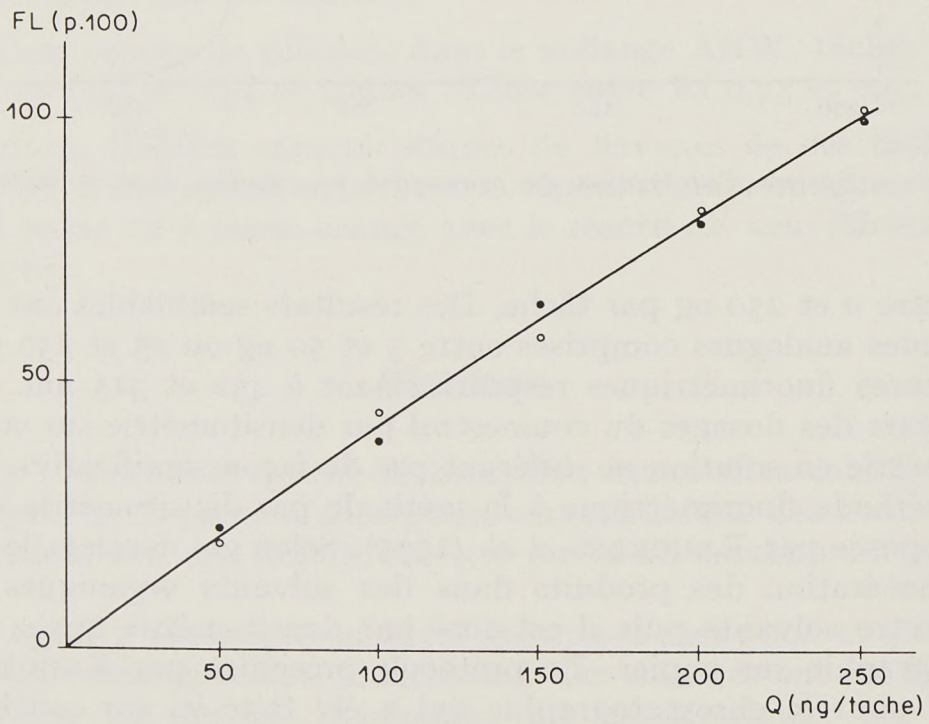

FIG. 3. — *Courbe d'étalonnage de la gamme de coumestrol*

Q = quantité de coumestrol en ng dans chaque tache.
F (p. 100) = fluorescence en pour cent des unités relatives de mesures. Détail du protocole de mesure dans le texte.

TABLEAU I

Reproductibilité des dosages du coumestrol séparé par chromatographie sur couche mince de cellulose

Dosage après extraction progressive sur colonne puis fluorimétrie du coumestrol en solution (Méthode A) soit après densitométrie après purification par partage entre solvants (Méthode B : d'après KNUCKLES *et al.*, 1975). Les résultats sont donnés en ppm de coumestrol : m, moyenne et σ, écart type correspondant.

Méthode	m	σ	$\frac{100\ \sigma}{m}$
A	76,1	2,3	3,0
B	73,9	2,7	3,5

Au cours de ces répétitions, on a contrôlé la reproductibilité du chargement des couches minces en introduisant un étalon interne dans le méthanol utilisé pour l'élution. Un flavonol, la myricétine ou 3, 5, 7, 3', 4',5'- hexahydroxyflavone a été finalement choisi parce qu'il est bien séparé par chromatographie des coumestanes à côté desquels il peut être dosé par densitométrie. Bien que cet échantillon commercial (FLUKA) n'ait pas été recristallisé, il ne contenait pas d'impuretés pouvant géner les dosages. Répétant 10 déterminations correspondant à des dépôts de 20 µl sur deux plaques différentes, on a obtenu des réponses moyennes relatives égales à 26,8 dans les deux cas avec des écarts types de 1,3 et 0,8 respectivement.

La méthode de dosage fluorimétrique a été ensuite appliquée au contrôle des teneurs en coumestrol et des coumestanes de différentes préparations protéiques traitées à l'aide de solvants.

La présence de coumestrol a été tout d'abord constatée par chromatographie dans les extraits par le pentane à 2 p. 100 de méthanol de plusieurs préparations protéiques de Luzerne. Au cours de ces analyses, on a observé aussi la présence de coumestrol dans les extraits par l'éther éthylique et par le méthanol préparés selon le protocole standard d'extraction progressive sur colonne. Ce dernier résultat confirme bien l'existence du phénomène de compartimentation du coumestrol décrit précédemment (MONTIES et RAMBOURG, 1978*a*). Ainsi, en plus de ces deux compartiments de coumestrol extractibles par l'éther éthylique et par le méthanol, il existerait, d'après l'observation, ci-dessus un troisième compartiment correspondant au coumestrol extractible par le mélange pentane-méthanol.

La présence de coumestrol dans les extraits pentane-méthanol peut cependant être expliquée *a priori* autant par un effet de compartimentation que par une dissolution sélective de traces de coumestrol par le méthanol mélangé au pentane. Pour tester cette hypothèse on a comparé deux protocoles d'extractions successives différant seulement par le premier solvant : mélange pentane à 2 p. 100 de méthanol dans un cas, pentane pur dans l'autre. Le tableau 2 présente le bilan de récupération du coumestrol dans les 9 fractions successives obtenues à l'aide des

trois solvants d'élution choisis successivement. On constate l'absence de coumestrol dans les extraits par le pentane pur et donc l'existence de seulement deux compartiments de coumestrol extractibles respectivement par l'éther éthylique et par le méthanol.

TABLEAU 2

Influence de la composition du premier solvant d'extraction progressive sur la compartimentation du coumestrol

Une même aliquote de protéines a été extraite selon deux séquences différentes d'extraction progressive :

Séquence A 1 : pentane + 2 p. 100 méthanol; 2 : éther éthylique; 3 : méthanol.
Séquence B 1 : pentane pur; 2 : éther éthylique; 3 : méthanol.

Pour chaque solvant, on a séparé trois fractions *a*, *b* et *c* dans laquelle le coumestrol a été dosé; résultats en pour cent du coumestrol total extrait.

Séquence et fractions	Solvant n° 1			Solvant n° 2			Solvant n° 3		
	1*a*	1*b*	1*c*	2*a*	2*b*	2*c*	3*a*	3*b*	3*c*
A.	2.6	1.9	3.2	18.5	7.1	5.8	57.5	1.7	0.7
B.	nd	nd	nd	4.0	0.6	nd	92.0	3.4	/

Nd : non détecté.

TABLEAU 3

Influence du lavage des protéines de luzerne sur leur teneur en coumestrol et en coumestanes

Teneurs exprimées en ppm de coumestrol et, entre parenthèse, en pourcentage du total (coumestrol + coumestanes) du produit non lavé sec.

Échantillon	Coumestrol		Coumestanes	
	Extrait MeOH	Total	Extrait MeOH	Total
Lavé	130 (8.1)	170 (10.6)	87 (5.4)	115 (7.2)
Non lavé	613 (38.4)	1 032 (64.6)	457 (28.6)	565 (3.4)

Pour établir un bilan des coumestanes dans les préparations protéiques nous avons donc utilisé par la suite la séquence d'extraction : pentane puis éther éthylique puis méthanol et enfin eau. Dans ces conditions, il est possible d'analyser la compartimentation du coumestrol et des coumestanes dans les extraits par l'éther éthylique et le méthanol. La compartimentation des flavonoïdes peut être analysée, à la suite et du même coup, dans les extraits méthanoliques et aqueux.

Ce protocole a été utilisé pour préciser l'influence de la technologie d'isolement des préparations protéiques sur leurs teneurs en coumestanes et flavonoïdes.

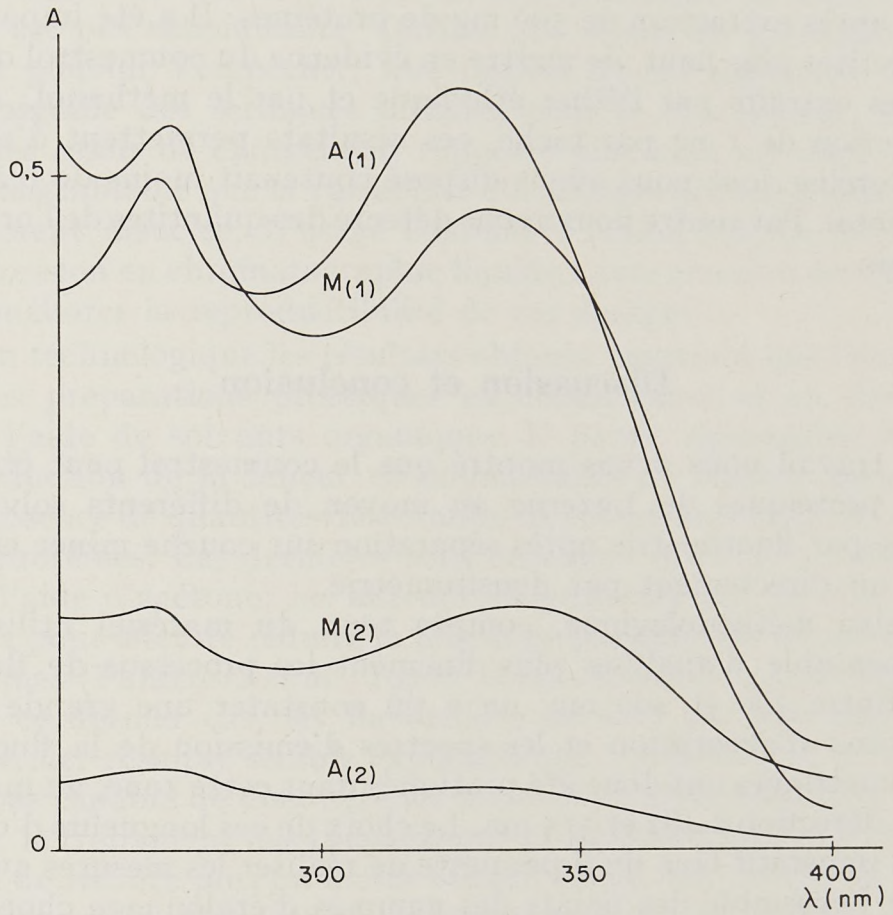

FIG. 4. — *Spectre d'absorption des extraits des isolats protéiques non lavés* (1) *et lavés* (2) *obtenus par le méthanol* (*M*) *et l'eau* (*A*).

Le même protocole d'extraction progressive sur colonne a été appliqué à des quantités égales d'isolats protéiques lavés ou non par l'éthanol. La comparaison du profil et de la hauteur relative des pics de chacun des spectres permet de vérifier la pureté relative des flavonoïdes extraits. La diminution des absorbances des extraits homologues (M_1, M_2, et A_1, A_2) illustre l'influence du lavage des isolats sur leurs teneurs en flavonoïdes.

Dans un premier temps, on a comparé des « isolats » de protéines thermoprécipités lavées ou non à l'éthanol. Dans le tableau 3 sont regroupés les résultats correspondant au coumestrol et aux coumestanes. Les coumestanes ont été identifiés sur couche de cellulose grâce à leur intense fluorescence bleue et leur Rf voisin de celui du coumestrol; ils ont été dosés aussi par densitométrie. On constate que le traitement à l'éthanol des isolats protéiques permet de réduire de près de 80 p. 100 la teneur totale en coumestanes et que, dans ces conditions, les proportions relatives coumestrol /coumestanes sont relativement inchangées. La figure 4 présente les spectres d'absorption des fractions extraites par le méthanol et par l'eau à partir de ces mêmes isolats. Le profil de ces quatre spectres qui présentent des maximums au voisinage de 270 et 330 nm et un minimum entre 290 et 300 nm

est très caractéristique des flavonoïdes. On constate donc, comme dans le cas des coumestanes, que le lavage à l'éthanol permet de réduire sensiblement la teneur en flavonoïdes totaux de l'isolat protéique. D'après les absorbances relatives de ces extraits, on peut estimer que le lavage élimine environ 75 p. 100 des flavonoïdes; on constate aussi qu'il n'affecte pas également les compartiments de flavonoïdes extractibles par le méthanol et par l'eau.

Enfin, dans un second temps, nous avons recherché la présence de coumestanes et de flavonoïdes dans les polyprotéines obtenues par précipitation à l'aide de polyélectrolytes. La recherche de coumestrol et de coumestanes a été faite par chromatographie après extraction de 500 mg de protéines. Il a été impossible, dans les conditions décrites plus haut, de mettre en évidence du coumestrol ou des coumestanes dans les extraits par l'éther éthylique et par le méthanol. Admettant un seuil de détection de 1 ng par tache, ces résultats permettent d'affirmer que le lot de polyprotéine dont nous avons disposé contenait moins de 0,2 ppm de coumestanes au total. Par contre nous avons détecté des quantités de l'ordre de 1 p. 100 de flavonoïdes.

Discussion et conclusion

Dans ce travail nous avons montré que le coumestrol peut être extrait des préparations protéiques de Luzerne au moyen de différents solvants, et qu'il peut être dosé par fluorimétrie après séparation sur couche mince et élution dans le méthanol ou directement par densitométrie.

Sur le plan méthodologique, compte tenu du matériel utilisé, il n'a pas semblé indispensable d'analyser plus finement les processus de fluorescence du coumestrol. Entre 300 et 400 nm, on a pu constater une grande ressemblance entre les spectres d'absorption et les spectres d'émission de la fluorescence. Les dosages fluorimétriques ont donc été pratiqués dans cette zone, au maximum d'excitation, avec détection à 462 et 515 nm. Le choix de ces longueurs d'onde de détection n'est pas impératif bien qu'il permette de réaliser les mesures avec une bonne sensibilité sur l'ensemble des points des gammes d'étalonnage choisies. Ces deux longueurs d'onde sont celles de filtres standard équipant l'appareil utilisé. Compte tenu de la position et de la forme des pics de fluorescence on peut supposer que ces détections ont lieu du côté des grandes longueurs sur la branche descendante du pic d'émission centré à 415 nm. Il en résulte la possibilité, mise à profit ici, de modifier la sensibilité des mesures en choisissant la longueur d'onde d'analyse de la fluorescence tout en conservant cependant la linéarité des courbes de dosage.

Dans ces conditions, les méthodes de dosage par fluorimétrie en phase liquide et par densitométrie sur couche mince permettent d'obtenir des résultats qui sont pratiquement équivalents : ce sont donc des commodités ou des objectifs particuliers qui permettent de préférer l'une à l'autre. Une conclusion analogue peut être tirée de la comparaison des protocoles d'isolement sur colonne, par extraction progressive (Monties et Rambourg, 1978) et en phase aqueuse, par partage entre solvants (Knuckles *et al.*, 1975).

Le protocole d'extraction sur colonne paraît préférable dans l'étude de la compartimentation : il est simple et rapide à mettre en œuvre. De plus, compte tenu du fort pouvoir solvant du méthanol, la méthode d'extraction sur colonne reste aussi préférable même lorsque l'étude de la compartimentation n'est pas envisagée; il suffit dans ces conditions d'utiliser la séquence simplifiée : pentane, méthanol, eau pour isoler les coumestanes en solution méthanolique. Le dosage des

coumestanes peut être fait ensuite après chromatographie sur couche mince. A ce propos, il convient de souligner que l'introduction de la chromatographie liquide haute pression sur phase inverse paraît susceptible d'améliorer la rapidité et peut être aussi la reproductibilité des différents dosages comparés dans ce travail. Les résultats publiés récemment par LOOKHART (1980) en ce domaine sont très encourageants. Ces dosages pourraient alors être faits, directement, par injection des extraits méthanoliques préalablement concentrés.

Nous avons contrôlé dans ce travail la qualité du chargement des chromatogrammes en utilisant un étalon interne. La reproductibilité de dépôt sur les couches minces n'est pas satisfaisante. Quelles que soient les précautions prises on constate, sans pouvoir l'empêcher, que parfois les microgouttes remontent et séchent sur l'aiguille des seringues utilisées pour le chargement. Le coefficient de variation des essais de chargement rapporté plus haut est donc relativement élevé. Il est vraisemblable que la variabilité des dosages présentés dans le tableau I lui est étroitement associée. L'usage combiné d'étalon interne et d'injecteur à boucle basse pression en chromatographie liquide haute pression devrait donc aussi permettre d'améliorer la reproductibilité de ces dosages.

Sur le plan technologique les résultats obtenus montrent que l'on peut réduire les teneurs des préparations protéiques en coumestanes et en flavonoïdes par « lavages » à l'aide de solvants organiques. Il paraît raisonnable de mettre en parallèle la réduction de la teneur en coumestanes de luzerne après lavage par l'éthanol et l'absence de quantités détectables de ces mêmes composés dans l'échantillon de polyprotéines. Ces dernières sont en effet « décolorées » par traitement au Soxhlet à l'aide d'acétone; les auteurs du protocole ont en effet montré que ce traitement a pour effet de réduire la teneur en polyphénols totaux de ces préparations protéiques (FELICIOLI *et al.*, 1978). Il est cependant surprenant de constater que, dans ce dernier cas, les flavonoïdes ne sont pas eux aussi totalement extraits. Ce dernier résultat semble explicable par l'utilisation d'acétone anhydre qui ne serait pas capable de dissoudre les flavonoïdes glycosidés trop hydrophiles. En conclusion, le traitement des préparations protéiques à l'aide de solvants pourrait permettre de réduire plus ou moins sélectivement leur teneur en polyphénols. Dans cette perspective, l'extraction de coumestrol par le pentane contenant de faibles quantités de méthanol mérite d'être discutée plus en détail.

Comparant deux gammes d'élution progressive sur colonne ne différant que par la composition du premier solvant, nous avons constaté que le pentane seul n'extrait pas de coumestanes et que, ensuite, les extractions par l'éther éthylique puis le méthanol permettent l'isolement de deux compartiments de coumestrol. Il y a bien dans ce cas compartimentation du coumestrol qui est extrait par deux solvants différents de façon chaque fois exhaustive car les concentrations des fractions successives diminuent. Au contraire, lorsque la première élution est pratiquée à l'aide de mélange pentane-méthanol, on constate la présence de traces de méthanol dans les différentes fractions successives; de plus, le phénomène de compartimentation entre éther éthylique et méthanol apparaît moins nettement. On peut admettre que ce dernier effet résulte de la forte polarité et donc du fort pouvoir solvant du méthanol vis-à-vis des coumestanes relativement polaires. Nous avons rapporté, par ailleurs, un phénomène analogue dans le cas des flavonoïdes : la présence de traces d'eau dans le méthanol ou dans l'acétone perturbe l'isolement des compartiments extractibles par ces solvants puis par l'eau. A la limite, les flavonoïdes et les coumestanes peuvent être extraits totalement au moyen d'une seule extraction par le méthanol contenant un faible pourcentage d'eau. En conclusion les résultats obtenus ici sur les préparations protéiques de Luzerne illustrent

la nécessité de contrôler rigoureusement les qualités des solvants et confirment de plus l'existence du phénomène de compartimentation des polyphénols dans les préparations protéiques de Luzerne.

Pour terminer il convient cependant de souligner que seul parmi les coumestanes et les flavonoïdes étudiés dans ce travail, le coumestrol a été purifié, caractérisé sans ambiguité et enfin dosé dans les extraits successifs notamment éther éthylique et méthanol. Ainsi, seule la compartimentation du coumestrol est établie ici sans ambiguité. Cette dernière conclusion permet de discuter à nouveau l'origine du phénomène de compartimentation. La formation de liaisons non covalentes et d'énergie différentes avec un nombre limité de sites récepteurs semble suffir pour expliquer l'existence d'effets de compartimentation révélables par extraction progressive à l'aide de solvants de polarité croissante (MONTIES, 1975, 1981). La formation de liaisons non covalentes a été postulée récemment pour expliquer des phénomènes voisins (ANDERSEN *et al.*, 1980). Comme l'addition de faibles quantités de composés polaires dans les solvants d'extraction suffit pour perturber la compartimentation du coumestrol des préparations protéiques de Luzerne, il paraît très peu vraisemblable que la formation de liaisons covalentes soit responsable de ce phénomène. Seules des liaisons covalentes suffisamment activées pour être particulièrement fragiles pourraient être mises en cause. Il faudrait alors expliquer pourquoi et comment ces sites de liaisons activées ne sont pas détruits par thermoprécipitation ou dénaturation des protéines par les solvants lors de leur isolement. Au contraire, des liaisons avec des polyosides, analogues à celles mises en jeu dans les associations entre certains polyphénols et des glycoprotéines du type β-lectines pourraient exister (MONTIES, 1981); ces sites seraient stables dans les conditions de préparations des protéines.

L'étude de l'origine du phénomène de compartimentation est donc intéressante à la fois sur le plan fondamental : elle conduit à la recherche chez les végétaux des récepteurs du type récepteurs hormonaux, et sur le plan pratique : elle permet de contrôler qualitativement et quantitativement la pureté des préparations protéiques alimentaires. Cette étude est poursuivie.

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Note

Une partie de ces résultats a fait l'objet d'une communication préliminaire intitulée : « Evidence of plant phenolics compartementation by progressive elution with solvants of increasing polarity » par l'un d'entre nous (B. M.) lors du Leopoldina Symposium : Cell compartmentation and metabolic channeling, 7.13 avril 1979, Halle, R.D.A.

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Summary

Fluorimetric and densitometric determinations of coumestrol applied to quality control of alfalfa leaf proteins

The characteristic absorption and fluorescence spectra of coumestrol (fig. 1 and 2) allow its fluorimetric determination in solution. After thin layer chromatographic separation and elution, linear fluorimetric responses were obtained in the range of 0 to 250 ng per spots (fig. 3).

Coumestanes and coumestrol, occurring in alfalfa leaf proteins, were estimated either after progressive elution with solvents and fluorimetry in solution either after phase partage or thin layer densitometry (tabl. 1).

The phenomenon of compartmentation between ethylether and methanol was observed for alfalfa protein coumestanes (tabl. 2); however, a complete extraction was obtained with pure methanol. Thermoprecipitated or polyproteine preparations were extracted with organic solvents such methanol or anhydrous acetone; significative reductions of coumestanes content (tabl. 3) but also of total flavonoïdes (fig. 4) were observed. The origin of the compartmentation phenomenon is discussed.

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Évolution de la microflore et de la capacité germinative des graines de Lupin blanc (*Lupinus albus L.*) au cours du stockage

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Résumé

Il n'existe que peu de données sur les conditions de stockage permettant d'assurer une bonne conservation des graines de Lupin blanc, pourtant considéré comme un des protéagineux de l'avenir.

Pour pallier ce manque d'informations, l'évolution au cours du stockage, de la microflore et de la capacité germinative, prises comme critères de qualité, a donc fait l'objet d'une étude préliminaire.

Des essais de stockage ont été réalisés à 15 et 25 °C sous des humidités relatives de 62,5-70,5-71,5-75 et 85 p. 100.

Les courbes isohydres d'évolution quantitative des moisissures et de la capacité germinative de graines initialement saines, mises en expérimentation telles quelles ou après réhumidification, ont permis de déterminer les durées maximales de bonne conservation des graines placées dans les conditions étudiées. D'après les résultats obtenus les graines de Lupin blanc présentent une bonne aptitude au stockage : par exemple, des graines saines ayant une teneur en eau initiale de 13 p. 100 se conservent bien à 15 °C durant plus de 20,5 mois aux humidités relatives égales ou inférieures à 75 p. 100, à 25 °C cette durée est encore voisine de 4 mois.

La durée de conservation des graines réhumidifiées est beaucoup plus courte, toutes autres conditions de stockage étant égales.

L'importance de la teneur en eau initiale est discutée car elle semble conditionner en partie la teneur en eau des graines en équilibre hydrique avec les atmosphères de stockage et se répercute sur l'évolution de la mycoflore et de la capacité germinative. On rappelle à ce propos quelques données récentes sur les rapports entre teneur en eau et configuration des endomembranes.

La mycoflore rencontrée est inventoriée et s'avère non spécifique du Lupin.

Mots clés : *stockage, microflore, germination, graine, Lupin blanc.*

Introduction

Le Lupin blanc (*Lupinus albus* L.) suscite vivement l'intérêt des cultivateurs, des éleveurs, des nutritionnistes et des technologistes de l'alimentation animale et humaine. En effet, c'est une plante dont les graines sont riches en protéines (environ 40 p. 100) avec une teneur élevée en acides aminés indispensables : lysine, leucine et thréonine (El-Dash et Sgarbieri, 1980).

Le Lupin étant considéré comme une des plantes protéagineuses de l'avenir, de nombreux travaux ont déjà été effectués ou sont en cours pour en préciser les techniques de culture, pallier la présence de facteurs antinutritionnels et élargir ses débouchés (farines, concentrés, extrudats).

Mais en regard de toutes ces recherches il semble qu'il y ait peu de travaux relatifs à la conservation des graines de Lupin après récolte. C'est la raison qui a motivé le présent travail limité, dans une première approche, à l'étude de l'évolution de la microflore et de la capacité germinative des graines de Lupin en fonction de conditions hydrothermiques déterminées de stockage.

D'une façon générale on sait que parmi les agents d'altération des graines figure en bonne place leur microflore qui, si elle vient à se développer, constitue une menace pour la qualité des stocks.

Il a été maintes fois constaté que le développement de la microflore des graines s'accompagne d'une dégradation de leur valeur d'utilisation : diminution de la faculté germinative et de la teneur en sucres, augmentation de l'acidité grasse, modification des propriétés rhéologiques et de la solubilité des protéines, formation éventuelle de toxines (Multon *et al.*, 1969; Christensen et Kaufmann, 1974).

Le développement de la microflore précédant l'évolution des autres caractéristiques de la graine, constitue en général un critère de qualité assez fiable. Son évolution est sous la dépendance, entre autres facteurs, des conditions écologiques du stockage : teneur en eau des graines, température et composition de l'atmosphère (Christensen et Kaufmann, 1965). L'isotherme de sorption de vapeur d'eau à 25 °C de la graine de Lupin blanc de la variété Kalina — illustrée par la figure 1 — (Multon et Gadet, 1977), donne en première approximation des indications sur les conditions hydriques de stockage permettant de maintenir ces graines à l'abri d'un développement microbien.

Aux humidités relatives inférieures à 62,5 p. 100 environ, il y a blocage de toute activité microbienne, soit à des teneurs en eau de la graine de Lupin inférieures à 10 p. 100 de la substance humide (s.h.*).

Sur des graines stockées en atmosphère libre ou ventilée, et se trouvant en équilibre hydrique avec des humidités relatives comprises entre 70 et 80 p. 100 (teneurs en eau des graines de 12 et 15 p. 100) les *Aspergillus* prédominent (Christensen et Kaufmann, 1974).

Aux humidités relatives de 85 à 90 p. 100 (teneur en eau des graines de Lupin de 18 à 22 p. 100), beaucoup de *Penicillium*, d'*Aspergillus*, de *Mucor* et de *Cladosporium* se développent.

Enfin, aux humidités relatives très élevées, supérieures à 90 p. 100, les moisissures prolifèrent, aussi bien les moisissures du champ à tendance phytopathogène que les moisissures de stockage ainsi que des levures et des bactéries.

(*) Dans la suite du texte, toutes les teneurs en eau sont exprimées en pourcentage de la substance humide.

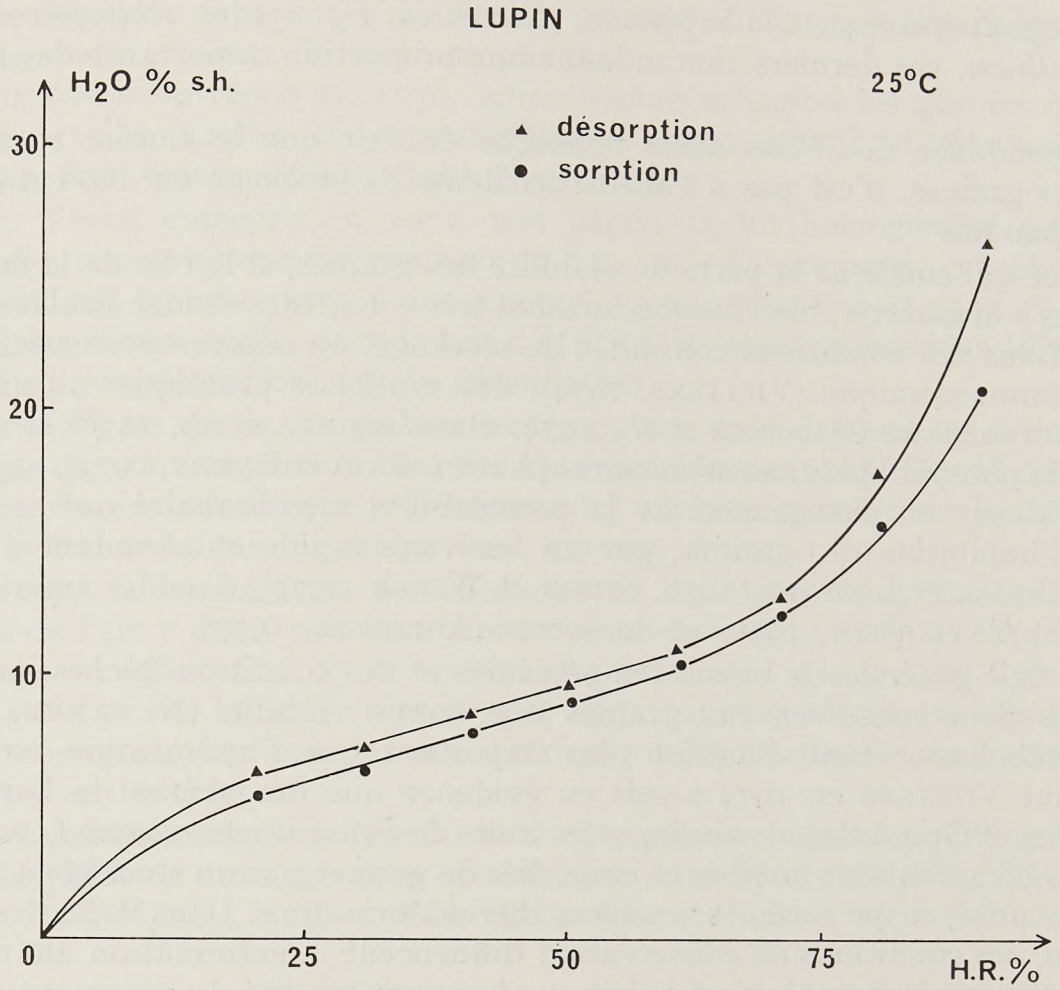

FIG. 1. — *Courbes isothermes, à 25 °C, de sorption (●) et de désorption (▲) de vapeur d'eau de graines de Lupin blanc*

Abscisse : humidité relative de l'air en p. 100.
Ordonnée : teneur en eau de la graine de Lupin en p. 100 de sa substance humide.

Cependant, dans la zone hydrique de développement des microorganismes, l'isotherme de sorption de vapeur d'eau de la graine ne fournit pas d'indication sur la vitesse de colonisation par les microorganismes, vitesse dont dépend pourtant la durée de bonne conservation de la graine.

Les bactéries n'étant pratiquement jamais à l'origine d'accidents de conservation, compte tenu du degré de siccité des graines dans les conditions les plus courantes de stockage, ce sont les germes fongiques, plus particulièrement les moisissures de stockage, qu'il importe de prendre en considération (CHRISTENSEN et KAUFMANN, 1974; MULTON *et al.*, 1969).

Dans le cas du Lupin, la mycoflore n'a suscité que fort peu de travaux. L'ISTA (**) (1968) a publié une liste des champignons phytopathogènes dont la présence a été signalée sur les semences de Lupin, mais ces champignons n'intéressent guère le stockage puisqu'ils appartiennent à la flore dite du champ qui ne peut se développer dans les conditions d'hydratation normale des graines après récolte. CHAMPION (1976 et 1977) a analysé la mycoflore de graines métropolitaines de Lupin, quelques mois après la récolte. A côté de parasites (*Fusarium, Botrytis cinerea, Macrophomina phaseoli, Pleiochaeta setosa*), il a mis en évidence

(**) ISTA : International Seed Testing Association; Association Internationale d'Essais de Semences.

des saprophytes comme *Cladosporium, Alternaria, Epicoccum, Rhizopus nigricans* et *Penicillium,* ces derniers contaminant une proportion importante des lots examinés.

L'abondance des *Penicillium* témoigne du fait que le Lupin, tout comme les autres graines, n'est pas à l'abri d'accidents de stockage en dépit d'une idée assez répandue.

En ce qui concerne la perte de viabilité des graines, si le rôle de la mycoflore n'est plus à démontrer, bien des mécanismes très complexes et mal élucidés entrent en jeu. Dans des conditions courantes de stockage, on observe des variations, au niveau chromosomique (Villiers, 1974), des synthèses protéiques au niveau de l'axe embryonnaire (Osborne *et al.*, 1974; Dell'Aquila *et al.*, 1978) et des oxydations de phospholipide smembranaires (Abdul-Baki et Baker, 1973), oxydations qui entraînent un changement de la perméabilité membranaire qui se traduit, lors de l'imbibition des graines, par un lessivage rapide et abondant d'électrolytes (Parrish et Leopold, 1978; Simon et Wiebe, 1975), d'acides aminés (Hendricks et Taylorson, 1976) et de sucres (Agrawal, 1977).

En règle générale, de basses températures et des conditions sèches sont indispensables pour conserver aux graines une bonne viabilité (Nakamura, 1975), leur métabolisme étant d'autant plus important que l'hydratation est élevée. Cependant Villiers en 1974 a mis en évidence que des akènes de Laitue var. Artic King et Grand Rapids ainsi que les fruits de *Fraxinus americana* L. se conservent très bien s'ils sont imbibés et empêchés de germer par un stockage à 25-30 °C et à l'obscurité, ce qui rend ces semences thermodormantes. Dans le cas des graines de Lupin, ces conditions de conservation influencent la germination ultérieure en intervenant sur la dureté, c'est-à-dire sur l'imperméabilité du tégument séminal. Une liaison très nette entre teneur en eau et dureté a été démontrée chez *Lupinus digitatus* (Gladstones, 1958), et *Lupinus consentinii* (Walton, 1977). Ainsi, chez *Lupinus digitatus,* l'imperméabilité est réversible si la teneur en eau reste comprise entre 11 et 14 p. 100, mais elle devient irréversible au-dessous de 9 p. 100. Walton (1977) a mis clairement en évidence que des graines stockées ayant une teneur en eau de 13,6 p. 100 gardent une bonne viabilité et une dureté minimale pendant 2 ans, ce qui se traduit par une émergence optimale.

Pour apporter un complément d'information à ces données d'incidence pratique, nous avons étudié le comportement de graines de Lupin blanc en fonction de diverses conditions de stockage, les unes reconnues généralement comme favorables à la conservation, et les autres beaucoup plus sévères, voisines de celles qui sont utilisées pour le vieillissement accéléré des semences (Delouche et Baskin, 1973) ou rencontrées au cours d'accidents de stockage dûs à des condensations par exemple.

Matériels et méthodes

Protocole expérimental

Les deux lots de Lupin blanc « Kalina » utilisés pour les essais successifs de stockage ont été récoltés l'un en 1977 et l'autre en 1978 par la Station d'Amélioration des Plantes Fourragères de l'I.N.R.A. de Lusignan (Vienne).

Avant la mise en expérimentation, chaque lot est homogénéisé pendant 30 mn

dans un mélangeur cubique préalablement stérilisé et tournant autour d'un de ses axes diagonaux à raison de 64 tours par mn.

Pour l'essai entrepris en 1978, après homogénéisation les graines de Lupin sont mises telles quelles en expérimentation sans séchage ni réhumidification préalables, leur teneur en eau étant de 10 p. 100.

Pour l'essai entrepris en 1977, une partie du lot homogénéisé est utilisée telle quelle (teneur en eau des graines : 12,8 p. 100); l'autre partie est réhumidifiée à 4 °C pendant 5 jours, jusqu'à des teneurs en eau voisines de 13,3 et 16,8 p. 100 par addition d'eau stérile, dans le but d'étudier l'action de conditions défectueuses de stockage (condensation) sur la germination.

Pour tous les essais, les graines sont réparties dans des sachets en mailles de polyamide qui sont suspendus dans des enceintes d'une capacité de 10 l, fermées de façon non étanche. A chaque prélèvement les analyses portent sur la totalité des graines contenues dans un sachet, soit environ 200 g.

En 1977, la température des enceintes est maintenue à 25 °C, les humidités relatives de l'air y étant constamment voisines de 62,5-75 et 85 p. 100. En 1978, les essais sont effectués à 15 et 25 °C à des humidités relatives de l'air égales à 70,5-75 et 85 p. 100, ainsi qu'à 71,5-75 et 85 p. 100 respectivement afin d'obtenir plus de renseignements dans la zone hydrique où les moisissures de stockage commencent à se développer.

Les humidités relatives désirées sont obtenues par mise en équilibre hydrique de l'atmosphère de stockage avec des solutions saturées de NH_4NO_3, NaCl et KCl en 1977, de NH_4NO_3, NaCl + KCl, NaCl, KBr et KCl en 1978 (tabl. 1).

Détermination de la teneur en eau des graines

Dix grammes de graines entières sont séchés en étuve à 130 °C pendant 15 à 16 heures. Les résultats sont exprimés en pourcentage de la substance humide. Les valeurs obtenues expérimentalement sont corrigées en tenant compte du fait que la méthode utilisée donne des valeurs supérieures de 0,4 p. 100 à celles obtenues avec la méthode fondamentale de référence (50 °C sous vide en présence de P_2O_5 — GUILBOT, MULTON et MARTIN, 1973).

Détermination quantitative et qualitative de la microflore des graines

Les microorganismes des graines sont dénombrés après avoir été mis en suspension dans une solution physiologique stérile : le diluant (NaCl : 8,5 g; bactopeptone : 1 g; tween 80 : 0,033 g; eau distillée sur verre : 1 000 ml; pH : 7).

Pour la préparation de la « suspension mère », 100 g de graines sont versés aseptiquement dans un ballon renfermant 300 ml de diluant stérile et 30 g de billes de verre de 3 mm de diamètre. Après une homogénéisation de 30 minutes réalisée à l'aide d'un agitateur à va-et-vient (à raison de 150 mouvements par minute) la suspension mère obtenue est soumise à l'analyse microbiologique proprement dite par la méthode classique des dilutions décimales suivies d'ensemencements sur milieux de culture en boîte de Pétri.

Les bactéries sont dénombrées sur la gélose « standard pour dénombrement » de l'Institut Pasteur (peptone : 5 g; extrait de levure déshydratée : 2,5 g; glucose : 1 g; agar-agar : 15 g; eau distillée sur verre : 1 000 ml; pH final : 7,0 ± 0,2) en présence d'actidione (solution stérile à 1 p. 1 000 ajoutée au milieu de culture prêt à couler à raison de 10 p. 100 de son volume). Les lectures sont effectuées après 72 h d'incubation à 30 °C ± 1.

Les levures et les moisissures sont dénombrées sur malt gélosé (extrait de malt : 20 g; agar-agar : 15 g; eau distillée sur verre : 1 000 ml; pH final : 7,0 ± 0,2) et sur milieu pour germes osmophiles (saccharose : 200 g; malt : 20 g; agar-agar : 15 g; eau distillée sur verre : 1 000 ml; pH final : 7 ± 0,2) en présence d'oxytétracycline (solution stérile à 1 p. 1 000 ajoutée

TABLEAU I

Organigramme des essais

Année de récolte	1977			1978							
Teneur en eau des graines à la récolte (p. 100 sh)	10			12,8							
Réhumidification	—			—						+	
Teneur en eau au début des essais (p. 100 sh)	10			12,8						13,3	16,6
Conditions de stockage :											
— Température °C	25			15	25	15	25	15	25	25	25
— Humidité relative (p. 100)	62,5	75	85	70,5	71,5	75	75	85	85	75	85
— Solution saline saturée utilisée (*)	NH_4NO_3	NaCl	KCl	NH_4NO_3	NaCl + KCl	NaCl	KBr	KCl	KCl	NaCl	KCl

(*) Rockland, 1960; Winston et Bates, 1960.

aux milieux de culture prêts à couler, à raison de 10 p. 100 de leur volume). Les lectures sont effectuées après 72 heures d'incubation à 24 °C pour les levures et après 7 à 10 jours pour les moisissures.

Les résultats sont exprimés en nombre de germes bactériens ou fongiques recensés par gramme de produit analysé.

En raison du rôle important qu'elles sont susceptibles de jouer dans la dégradation des graines au cours du stockage, les moisissures ont été déterminées avec précision jusqu'au rang d'espèce.

Détermination de la capacité germinative

Les essais de germination sont réalisés par une méthode rapide. Les graines sont rincées à l'eau distillée stérile avant d'être déposées en boîtes de Pétri de 18,5 cm de diamètre, sur deux papiers filtre Whatmann n° 1, humidifiés par de l'eau stérile. Chaque essai comporte 4 boîtes de 50 graines, soit 200 graines.

Les boîtes de Pétri ainsi préparées sont placées dans une pièce thermostatée à 20 ± 1 °C en lumière diffuse. Les dénombrements de graines germées sont effectués au deuxième jour de l'essai. On considère qu'une graine a germé quand la radicule a percé les téguments séminaux.

Les résultats sont donnés en pourcentage de graines germées par rapport au nombre de graines mises en essai. Il faut remarquer que les conditions utilisées n'étant pas exactement celles préconisées par les règles de l'I.S.T.A. (*) (1976) nous ne parlons ici que de capacité et non de faculté germinative. Les lectures de germination étant réalisées au deuxième jour, les valeurs trouvées peuvent être inférieures à celles obtenues en appliquant les règles qui indiquent un dénombrement au quatrième puis au dizième jour. Ceci n'est vrai que pour des lots « paresseux » évoluant très lentement. Ainsi, des graines de Lupin conservées à 15 °C sous H.R. (**) de 70 p. 100 germent à 69,5 p. 100 au deuxième jour, à 98 p. 100 au quatrième jour et à 99 p. 100 au dixième jour. Par contre, un lot conservé à 25 °C sous HR. de 71,5 p. 100 donne les résultats suivants : 3-43 et 47 p. 100 de germination aux deuxième, quatrième et dixième jours.

Résultats obtenus

1. — *Évolution de la teneur en eau des graines au cours des essais de stockage*

La teneur en eau augmente de façon significative dans tous les lots au début du stockage sauf sous l'H.R.(**) de 70,5 p. 100 à 15 °C.

— Dans les lots de graines initialement sèches (fig. 2 (*a*) et 2 (*b*)) :

à 15 °C, sous les H.R. de 70,5 et 75 p. 100, les teneurs en eau des graines en équilibre hydrique avec l'atmosphère de stockage sont voisines de 13 et 15 p. 100 respectivement.

à 25 °C, sous H. R. de 62,5 p. 100, la teneur en eau atteint presque 12 p. 100 à l'équilibre. Sous H. R. de 75 p. 100, en 1977, la teneur en eau des graines se stabilise vers 15 p. 100 alors qu'en 1978, après avoir atteint ce même niveau au cours des deux premiers mois de stockage, elle s'abaisse et se stabilise vers 14 p. 100. Enfin, sous H. R. de 85 p. 100, la teneur en eau s'élève jusqu'à des valeurs quelque peu supérieures à 20 p. 100 en 1977 et voisines de 19 p. 100 en 1978.

(*) « Between paper » (BP), 20 °C, obscurité, dénombrement des plantules aux quatrième et dixième jours.

(**) H.R. : Humidité relative.

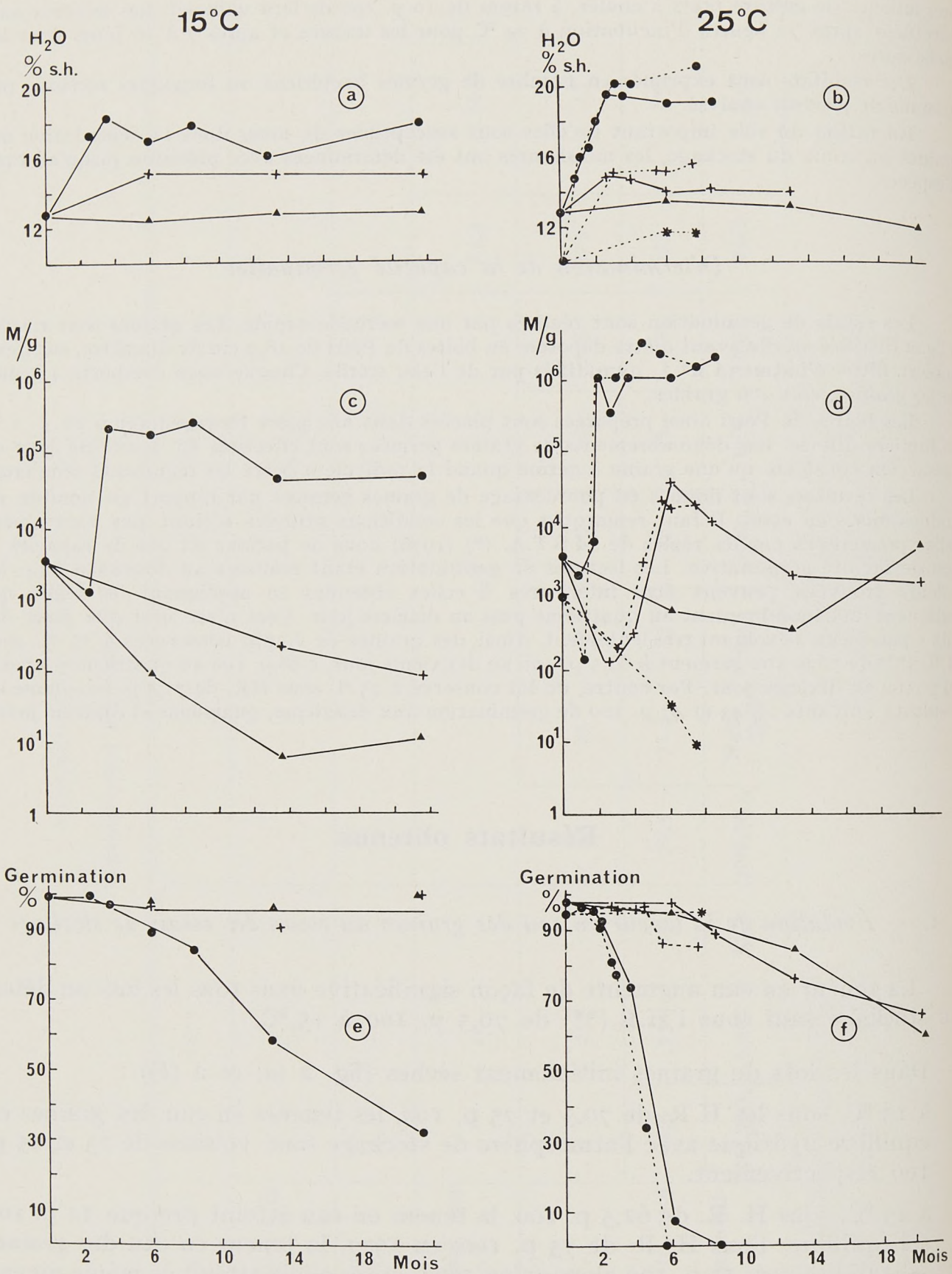

FIG. 2. — *Évolution de la teneur en eau, de la mycoflore et de la capacité germinative de graines de Lupin blanc stockées à 15 et 25 °C sous des humidités relatives de :* 62,5 (*), ≃ 71 (▲), 75 (+) et 85 (●) p. 100

..... récolte 1977.
——— récolte 1978.

Abscisse : durée de stockage en mois.
Ordonnée : — teneur en eau en p. 100 s.h. (fig. 2*a* et 2*b*);
— nombre de germes de moisissures par gramme de graines (fig. 2*c* et 2*d*);
— capacité germinative en p. 100 (fig. 2*e* et 2*f*).

Dans l'ensemble, les teneurs en eau atteignent donc des valeurs un peu plus élevées que celles qui sont indiquées par la courbe de sorption de vapeur d'eau (fig. 1), ce qui peut sans doute s'expliquer par le fait que cette dernière a été établie par la méthode rapide à l'étuve conditionnée (MULTON et GUILBOT, 1967) pour laquelle la durée de mise en équilibre hydrique est beaucoup plus courte que celle effectivement réalisée au cours de stockages expérimentaux.

— Dans les lots de graines réhumidifiées, puis stockées à 25 °C (fig. 3*a*) :

sous H. R. de 75 p. 100, la teneur en eau des graines atteint un niveau légèrement supérieur à 16 p. 100, soit environ 1 p. 100 de plus que pour les graines non rehydratées.

De même, sous H. R. de 85 p. 100, la teneur en eau des graines réhumidifiées à l'équilibre est presque de 21 p. 100, soit près de 2 p. 100 de plus que pour les graines initialement sèches.

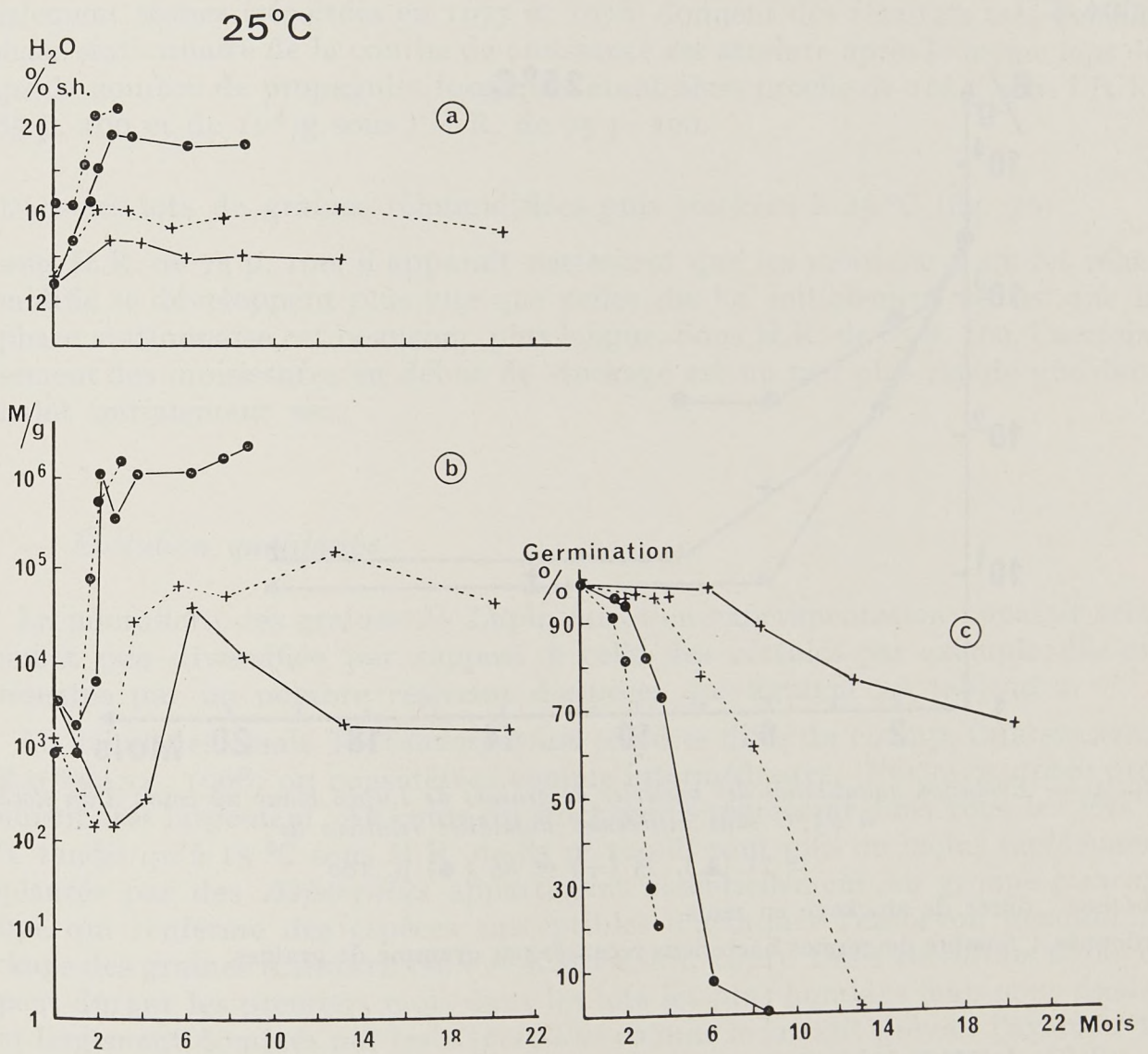

FIG. 3. — *Évolution, à 25 °C, de la teneur en eau, de la mycoflore et de la capacité germinative de graines de Lupin blanc initialement sèches* ——— *ou humides* ---------- *sous des humidités relatives de :* 75 (+) et 85 (●) p. 100

Abscisse : durée de stockage en mois.

Ordonnée : — teneur en eau en p. 100 (fig. 3*a*);
— nombre de germes de moisissures par gramme de graines (fig. 3*b*);
— capacité germinative en p. 100 (fig. 3*c*).

2. — *Évolution de la microflore des graines*

2.1. — *Évolution quantitative*

2.11. *Bactéries*

A la récolte, les graines de Lupin examinées sont très pauvres en bactéries (quelques centaines à quelques milliers par gramme) par rapport aux autres graines protéagineuses et surtout par rapport aux céréales qui en supportent plusieurs millions.

Au cours des essais de stockage, le nombre de bactéries diminue dans tous les lots : ainsi en 1977, au cours des cinq premiers mois de stockage, il est divisé par 100 sous H. R. de 85 p. 100 et par 40 sous H. R. de 75 p. 100. A titre d'exemple, l'évolution des bactéries dans les graines de 1978 stockées à 25 °C est illustrée par la figure 4.

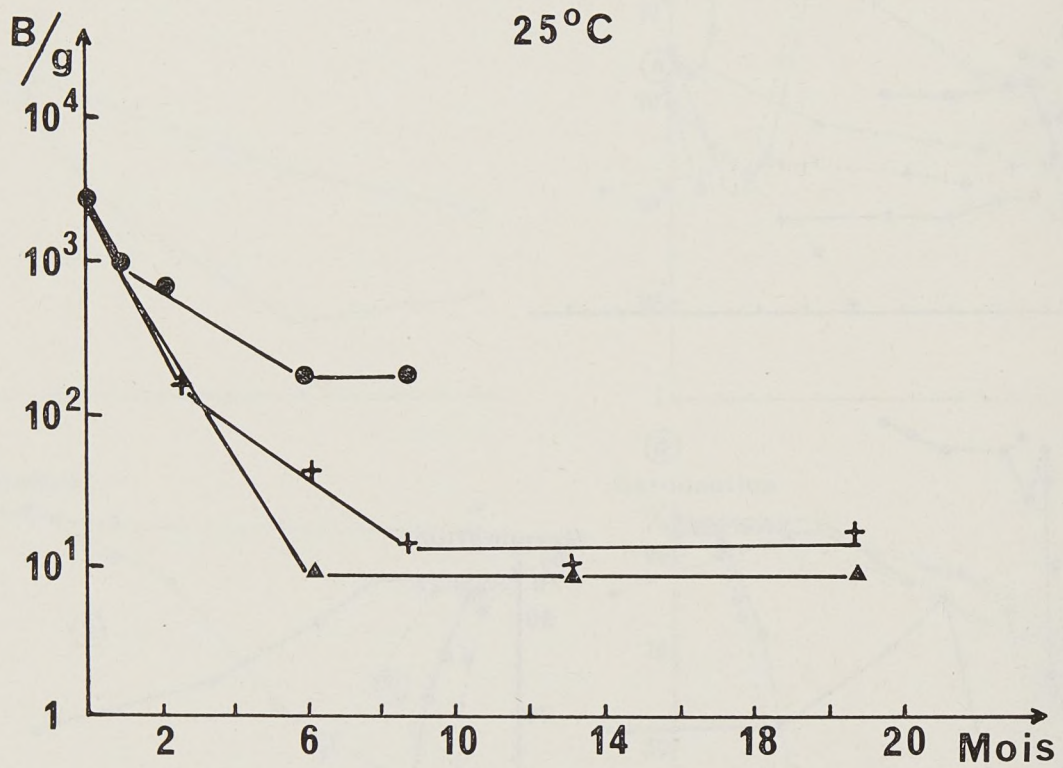

FIG. 4. — *Évolution quantitative des bactéries de graines de Lupin blanc au cours d'un stockage à 25 °C sous différentes humidités relatives de :*

≃ 71 (▲), 75 (+) et 85 (●) p. 100

Abscisse : durée de stockage en mois.

Ordonnée : nombre de germes bactériens recensés par gramme de graines.

2.12. *Germes fongiques*

Le nombre de germes fongiques, presqu'exclusivement de moisissures, diminue au début du stockage dans tous les lots; cette diminution est due à la régression de la flore du champ. A l'intérieur de la zone hydrique de développement des moisissures, il croît ensuite d'autant plus rapidement que la teneur en eau et la température sont plus élevées.

— Dans les lots de graines initialement sèches :

à 15 °C (fig. 2*c*), sous les H.R. voisines de 71 p. 100 et 75 p. 100, le nombre de germes fongiques régresse pendant la durée des essais. Sous l'H.R. de 85 p. 100, il croît lentement pour atteindre un palier voisin de 10^5 germes par gramme de graines; cette augmentation est due à la multiplication d'*Aspergillus* du groupe *glaucus*.

à 25 °C (fig. 2*d*) sous l'H.R. voisine de 62,5 p. 100, la mycoflore des graines régresse tout au long du stockage. Sous l'H.R. voisine de 71 p. 100, il y a augmentation des moisissures après plus d'un an des tockage; ce sont des *Aspergillus* du groupe *glaucus* donnant, sur malt gélosé, une forte proportion de colonies « intramatricielles » (*) qui n'ont jamais été observées lors d'études réalisées avec d'autres graines protéagineuses : pois, féverole, soja, haricot.

Sous les H.R. voisines de 75 et 85 p. 100, les essais de stockage des graines initialement sèches (récoltées en 1977 et 1978) donnent des résultats très voisins, la phase stationnaire de la courbe de croissance est atteinte après le même laps de temps, le nombre de propagules fongiques étant alors proche de 10^6/g sous l'H.R. de 85 p. 100 et de 10^4/g sous l'H.R. de 75 p. 100.

— Dans les lots de graines réhumidifiées puis stockées à 25 °C (fig. 3*b*) :

sous H.R. de 75 p. 100, il apparaît nettement que les moisissures du lot réhumidifié se développent plus vite que celles du lot initialement sec et que la phase stationnaire est beaucoup plus longue. Sous H.R. de 85 p. 100, l'accroissement des moisissures en début de stockage est un peu plus rapide que dans le lot initialement sec.

2.2. — *Évolution qualitative*

La microflore des graines de Lupin mises en expérimentation apparaît relativement peu diversifiée par rapport à celle des céréales par exemple; elle est représentée par un nombre restreint d'espèces qui figurent au tableau 2.

Au début des essais, les *Cladosporium* (espèces dites du champ, Christensen et Kaufmann, 1968; ou considérées comme intermédiaires, (Pelhate, 1968) prédominent très largement. Au cours du stockage ils régressent dans tous les lots à 25 °C tandis qu'à 15 °C sous H.R. de 85 p. 100 ils sont plus ou moins rapidement supplantés par des *Aspergillus* appartenant essentiellement au groupe *glaucus*, groupe qui renferme des espèces susceptibles d'attaquer l'embryon pendant le stockage des graines (Christensen et Kaufmann, 1974). Des *Penicillium* se développent durant les premiers mois dans les lots les plus humides mais sont finalement largement dominés par les *Aspergillus* comme le laissait prévoir l'examen de l'isotherme de sorption de vapeur d'eau.

Aux faibles humidités relatives, toutes les espèces régressent, mais après deux ans de stockage, la flore du champ est encore bien représentée.

(*) Les colonies se développent et fructifient au sein du milieu gélosé et non à sa surface.

TABLEAU 2

Mycoflore des graines de Lupin blanc mises en expérimentation

	Années	
	1977	1978
Espèces du champ et intermédiaires		
Acremonium bacillisporum (Onions et Barron) W. Gams.		+
Alternaria		
tenuissima (Fr.) Wiltshire		+
tenuis Nees	+	
Aureobasidium pullulans (De Bary) Arn.	+	+
Botrytis cinerea Pers. ex Fr.	+	
Chrysosporium pannorum (Link) Hughes		+
Cladosporium		
cladosporioides (Fresen) De Vries	+	+
herbarum (Pers) Link	+	+
Mycelia sterilia	+	+
Paecilomyces varioti Bain	+	
Verticillium albo-atrum Reinke et Berthold	+	
Espèces de stockage		
Aspergillus		
candidus Link	+	+
fumigatus Fr.		+
groupe glaucus		
amstelodami (Mang.) Thom et Church		+
chevalieri (Mang.) Thom et Church	+	
repens de Bary	+	+
ruber (Spieck et Brenner) Thom et Church	+	+
ochraceus Wilh.	+	
resctrictus Smith	+	+
sydowi (Bain et Sart.) Thom et Church	+	+
versicolor (Vuill.) Tiraboschi	+	
Penicillium		
brevicompactum Dierckx	+	+
capsulatum Raper et Fennell	+	+
citrinum Sopp		+
cyclopium Westl.		+
frequentans Westl		+
lilacinum Thom		+
notatum Westl.		+
stoloniferum Thom		+
vinaceum Gilman et Abbott	+	
spp (lanata et velutina)		
Levures	+	+

3. — *Évolution de la capacité germinative*

Au cours des essais, les graines conservent d'autant mieux leur aptitude à germer qu'elles sont initialement plus sèches et que l'humidité relative de l'atmosphère de stockage et la température sont plus basses.

— Dans les lots de graines initialement sèches :

à 15 °C (fig. 2*e*), sous H.R. de 70,5 p. 100 comme sous H.R. de 75 p. 100 la capacité germinative ne varie pas de façon significative au cours des essais (20,5 mois). Sous H.R. de 85 p. 100 elle commence à se dégrader après le deuxième mois, puis de façon plus accélérée après le quatrième mois.

à 25 °C (fig. 2*f*), sous H.R. de 62,5 p. 100 la capacité germinative reste élevée pendant toute la durée du stockage. Sous H.R. de 71,5 p. 100 elle ne décroît notablement qu'après 8 mois et cependant reste encore importante après 14 mois de stockage (85 p. 100). Sous H.R. de 75 p. 100 la capacité décroît plus tôt; elle diminue après 4 mois pour les graines de 1977 et après 6 mois pour celles de 1978 où elle est encore voisine de 80 p. 100 après un an (la différence entre les deux années pourrait être due à une teneur en eau légèrement inférieure dans le deuxième cas). Sous H.R. de 85 p. 100, le phénomène de dégradation s'accuse fortement, la diminution de la capacité germinative est de plus en plus rapide après le deuxième mois, les courbes d'évolution établies avec les graines de 1977 et 1978 sont très voisines.

— Dans les lots de graines réhumidifiées puis stockées à 25 °C (fig. 3*c*) :

sous H.R. de 75 p. 100 la capacité germinative s'abaisse rapidement à partir du quatrième mois, elle est nulle au quatorzième mois alors que pour les graines initialement sèches, elle est encore proche de 75 p. 100.

sous H.R. de 85 p. 100 l'aptitude à germer diminue encore plus vite : 2 mois après le début des essais elle n'est plus que de 80 p. 100, tandis que dans les mêmes conditions de stockage, celle des graines initialement sèches ne s'abaisse à cette valeur qu'un mois plus tard.

Discussion

Bien que les essais aient été effectués en petit nombre, les résultats obtenus fournissent quelques indications pratiques sur la durée de bonne conservation des graines en fonction des conditions de stockage réalisées.

Le niveau de peuplement des graines en espèces fongiques de stockage au-delà duquel elles sont considérées comme dégradées, est provisoirement fixé à 10^5 germes par gramme pour les céréales. A partir de ce seuil les moisissures de stockage commencent à être visibles à l'œil nu (Poisson et Cahagnier), il précède la détérioration des qualités technologiques du grain et est important pour l'appréciation de sa valeur commerciale.

En adoptant, pour l'instant, cette même limite et en tenant compte des courbes isohydres d'évolution des moisissures dans les lots de graines de Lupin initialement sèches, il est possible d'évaluer les durées probables de bonne conservation des graines dans les conditions hydrothermiques de stockage réalisées. Ces durées sont consignées dans le tableau 3. On peut considérer que ce sont des durées maximales puisque les graines mises en expérimentation étaient en bon état au début des essais et n'avaient pas été, au préalable, le siège d'un développement de la flore de stockage (Christensen et Kaufmann, 1968; Poisson et Cahagnier, 1979). Les performances des graines récoltées en 1977 et en 1978 sont très voisines, ce qui conforte la valeur des courbes établies. Il est à noter que les renseignements

fournis par les essais effectués sous H.R. de 71 p. 100 sont particulièrement intéressants, en effet la teneur en eau des graines s'y trouve d'emblée en équilibre hydrique avec l'atmosphère de stockage, donc, dans ces mêmes conditions de stockage, toute graine ayant une teneur en eau initiale inférieure se conservera plus longtemps.

Dans les lots de graines réhumidifiées, la multiplication des moisissures est plus rapide et le palier de leur courbe d'évolution plus élevé que dans les lots secs. Cette accélération du développement fongique est due non seulement à une teneur en eau initiale plus élevée, mais aussi au fait que la réhumidification (simulant un accident sévère de refroidissement et de condensation) a modifié les caractéristiques de sorption des graines comme en témoignent les courbes d'évolution de la teneur en eau; un phénomène analogue, par ailleurs, a été mis en évidence sur céréales et graines oléagineuses (POISSON et CAHAGNIER, 1979). L'influence de la réhydratation apparaît plus nettement sous H.R. de 75 p. 100 que sous H.R. de 85 p. 100 où les moisissures se trouvent plus près des conditions optimales de leur développement et sont donc moins sensibles à des modifications de faible amplitude.

En ce qui concerne la nature de la mycoflore, bien que le nombre d'espèces recensées soit relativement peu élevé, on doit noter la présence inhabituelle sur les graines de quelques unes d'entre elles.

Ainsi peut-on dire d'*Acremonium bacillisporum* qu'il est un hôte fréquent des sols, soit sous les feuillus, soit sous les conifères ou encore des sols à haute teneur saline; il a été isolé également de la pulpe de noix au Nigeria, mais ne semble pas avoir été recensé sur les graines.

De même, *Chrysosporium pannorum*, connu surtout comme dermatophyte et comme champignon du sol, a été isolé également d'aliments congelés, de viande stockée, et à partir de pourriture de sapin, mais il n'a pas été signalé comme séminicole.

Verticillium albo-atrum est un organisme pathogène notoire de nombreuses plantes cultivées; sa présence sur les graines en stockage ne suffit pas cependant à préjuger de son rôle de parasite du Lupin.

Parmi les espèces de stockage, trois espèces de *Penicillium* retiennent l'attention. *Penicillium capsulatum* isolé, par ailleurs, d'un lot de savonnettes altérées par des moisissures, en provenance de Dakar, a été signalé également sur des substrats variés, instruments d'optique, toile de tente, poisson séché... mais aucun auteur ne mentionne sa présence sur des graines.

Penicillium lilacinum, mis en évidence surtout comme entomopathogène et comme champignon du sol, est également mentionné sur de la pulpe de bois et sur des racines. Sa présence sur les graines semble assez peu fréquente et mérite d'être notée; il en est de même pour *Penicillium vinaceum* dont l'habitat se situe principalement dans le sol.

On peut donc noter que si les espèces fongiques recensées sur les graines de Lupin sont relativement peu nombreuses, en revanche, elles constituent une entité peu banale, aussi bien en ce qui concerne la flore du champ que la flore de stockage, sans qu'il soit possible cependant de parler de flore véritablement spécifique de ces graines.

En ce qui concerne la capacité germinative il est également possible de prévoir la durée de conservation d'un lot, compte tenu des courbes isohydres de son évolution.

TABLEAU 3

Durée, en mois, de bonne conservation des graines de Lupin blanc d'après les deux critères examinés

Conditions de stockage		Critères		
			capacité germinative	
température °C	H.R. p. 100	microbiologie	80 p. 100	60 p. 100 (*)
15	70,5	> 20,5	> 24,5	> 24,5 (**)
	75	> 20,5	> 20,5	> 20,5 (**)
	85	≃ 3	8,5	13
25	67,5 (1977)	> 10	> 10	< 10 (**)
	71,5 (1978)	> 20,5	≃ 14,5	> 20,5 (**)
	75 (1977)	> 10	> 10	> 10 (**)
	75 (1978)	> 20,5	≃ 11,5	> 20,5 (**)
	85 (1977)	≃ 2	2,5	≃ 4
	85 (1978)	≃ 2	3	≃ 4

(*) Ce seuil correspond approximativement au seuil commercial de 80 p. 100 déterminé par la méthode ISTA.

(**) Durée de l'essai.

Les durées de stockage pendant lesquelles la capacité germinative des graines initialement sèches reste supérieure à 80 p. 100 et 60 p. 100 sont consignées dans le tableau 3.

Ainsi, les graines de Lupin blanc ayant une teneur en eau initiale proche de 13 p. 100 (12,8 p. 100) conservent à 15 °C une capacité germinative élevée de plus de 80 p. 100 (la lecture étant faite à 2 j) pendant plus de 21 mois (durée des essais) comme à 25 °C sous des H.R. voisines de 71 et 75 p. 100, donc dans des conditions de stockage considérées comme médiocres et même mauvaises. Ces résultats sont en accord avec ceux de WALTON (1977).

La diminution plus rapide de la capacité germinative des graines préalablement réhumidifiées souligne l'importance de l'état initial des lots. La réhumidification doit intervenir d'une part sur l'augmentation des activités enzymatiques propres des graines donc sur l'accélération du vieillissement et d'autre part sur le développement de moisissures susceptibles d'attaquer le germe, phénomènes concourant à la dégradation de l'aptitude à germer.

Que les graines soient initialement sèches ou réhumidifiées, la diminution de la capacité germinative s'accélère en général à la période correspondant au début de la phase stationnaire de la « courbe de croissance » des moisissures. Une telle relation entre le développement des moisissures et la dégradation de la valeur d'utilisation de graines protégées par des téguments, notamment d'akènes de tournesol, a déjà été mise en évidence (GUENOT et POISSON, 1974).

Les teneurs en eau observées au cours des expérimentations sont généralement inférieures ou voisines de 20 p. 100. Dans ces conditions SIMON et WIEBE (1975) estiment que les endomembranes cellulaires sont très poreuses, leur conformation en bicouche étant incomplète si l'on se réfère aux résultats obtenus à l'aide de systèmes artificiels lécithine — eau ou bien d'extraits de lipides du cerveau humain (LUZZATI et HUSSON, 1962; CHAPMAN, WILLIAMS et LADBROOKE, 1967). Cette porosité accrue et l'évolution concomitante de la microflore expliquent que lors de l'imbibition des graines il y ait un lessivage important dans le milieu extérieur de petites molécules directement accessibles pour la microflore présente sur et dans la graine ou bien dans le milieu environnant.

Conclusion

Cette étude a été entreprise pour pallier le manque de données relatives à la conservation des graines de Lupin. C'est un travail préliminaire qui a porté sur quelques essais de stockage de graines de Lupin blanc, de bonne qualité (utilisées telles quelles ou réhumidifiées en laboratoire) effectués à 15 °C et 25 °C en atmosphère non ventilée, sous des humidités relatives s'échelonnant de 62,5 p. 100 à 85 p. 100, soit dans des conditions considérées en général comme bonnes, médiocres et mauvaises.

L'évolution des critères de qualité, importance et nature de la microflore et valeur de la capacité germinative, a été suivie en fonction des caractéristiques de stockage.

La microflore des graines mises en expérimentation s'est révélée relativement peu diversifiée et non spécifique, et ne laissait donc prévoir aucune réaction particulière.

En effet, dans les conditions réalisées, les résultats obtenus concordent avec ceux de travaux antérieurs du même type effectués aussi bien sur céréales que sur graines oléagineuses et protéagineuses.

Les bactéries régressent au cours du temps, il en est de même des moisissures sous l'humidité relative de 62,5 p. 100 ainsi que sous l'humidité relative de 70,5 p. 100 à 15 °C. Dans les autres conditions, le développement des moisissures est d'autant plus précoce et plus important que l'humidité relative et la température de stockage sont plus élevées. Les moisissures du champ, initialement prédominantes, diminuent quelles que soient les conditions expérimentales réalisées et lorsqu'il y a une augmentation des moisissures celle-ci est due à la multiplication de *Penicillium* et surtout d'*Aspergillus*.

La capacité germinative diminue au cours des essais dans toutes les conditions réalisées : très lentement dans la zone hydrothermique de non développement des moisissures, et d'autant plus rapidement que la température et l'humidité relative sont plus élevées.

A partir des courbes d'évolution des critères : importance de la mycoflore et capacité germinative, il est possible d'obtenir des indications sur les durées maximales de bonne conservation de graines placées dans les conditions des essais. Ainsi, des graines initialement en bon état, ayant une teneur en eau proche de 13 p. 100, stockées à 15 °C sous des humidités relatives égales ou inférieures à 75 p. 100, voient leur microflore diminuer tout en conservant un pouvoir germinatif élevé pendant une période au moins égale à 20,5 mois (durée des essais); pour les graines ayant une teneur en eau voisine de 15 p. 100 (seuil de séchage fréquemment

adopté) la durée de bonne conservation est de 16 mois au moins. Dans ces mêmes conditions hydriques la durée de bonne conservation est encore voisine de 4 mois à 25 °C. La graine de Lupin, d'après les critères considérés, possède donc une bonne aptitude au stockage, comme d'ailleurs le Pois et la Féverole.

La diminution de la valeur d'utilisation de graines de moindre qualité, comme les graines ayant été réhumidifiées au froid, est plus rapide dans les mêmes conditions de stockage, ce qui souligne l'influence de l'état initial des graines sur leur aptitude au stockage. En ne considérant que la teneur en eau des graines et son évolution au cours du stockage, on peut insister sur le fait que l'hydratation intervient à deux niveaux, (non seulement sur le développement des microorganismes mais aussi sur les variations de la capacité germinative) : la teneur en eau des graines au moment du stockage et la teneur en eau atteinte au cours du stockage qui dépend en outre de la nature de la graine et de l'humidité relative. Il est donc indispensable de mieux comprendre comment l'état hydrique initial des graines conditionne leur évolution au cours du stockage. Un travail de recherche sera entrepris prochainement dans cette optique.

L'étude présentée ne constituant qu'un sondage préliminaire à l'échelle du laboratoire, mais la zone de stabilité étant néanmoins cernée, il serait intéressant de préciser et de compléter les indications relatives à la durée de conservation des graines de Lupin blanc, d'une part en effectuant des essais de stockage en conditions réelles et d'autre part en étudiant l'influence de l'état de la graine au début du stockage.

Sur le plan méthodologique, la réhumidification des graines à basse température, en modifiant les caractéristiques de sorption, ne donne pas des résultats extrapolables aux graines récoltées à des teneurs en eau équivalentes comme on le supposait; par contre ce mode opératoire, qui permet de simuler un accident de conservation, en mettant en évidence la dégradation plus rapide de la capacité germinative au cours du stockage, apporte un élément nouveau.

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Summary

Microflora and germinative capacity evolution of white Lupine seeds during stockage (Lupinus albus L.)

There is no detailed data for storage conditions allowing a good conservation of white lupine seed which is considered as the proteaginous of the future.

To overcome this lack of information, we have followed the microflora and germinative faculty evolution, during the time of storage.

Very precise storage tests have been done at 15 and 25 °C under relative humidities (R.H) 63.5-70.5-71.5-75 and 85 p. 100.

The time for good conservation of seeds placed under the studied conditions was determined from the isohydric curves of the quantitative mould evolution and the germinative capacity of (initially healthy or rehydrated) seeds. For example healthy seeds with 13 p. 100 initial water content have good conservation at 15 °C during more than 20.5 months under relative humidities equal or less than 75 p. 100; at 25 °C this duration is still near to 4 months.

The conservation of rehydrated seeds is much shorter, under equal storage conditions.

The importance of initial water content is discussed because it affects the adjustment of seeds to the storage atmosphere. Initial water content has a great influence on mycoflora evolution and germinative capacity.

The mycoflora we have recognized is not specific to seed lupine.

The white lupine seed has a very good storage aptitude, but we must account for its initial state which determines partially its future, specially its microbiological quality andi ts use value. Some recent data are given concerning the relations between water content and endomembranes configuration.

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Vinification par macération carbonique

IX. — Élaboration de vins rosés

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avec la collaboration technique de

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Résumé

La vinification par macération carbonique a été utilisée pour l'élaboration des vins rosés de *Carignan*. Les vins obtenus par cette technique sont jugés supérieurs, sur le plan organoleptique, à ceux issus d'un pressurage direct de vendange; ils sont d'un niveau proche de celui des vins de saignée.

Cependant, une maîtrise précise de la température et de la durée de la première phase de fermentation est nécessaire de façon à obtenir des produits suffisamment aromatiques et à faible teneur en substances phénoliques.

Mots clés : *vin rosé, macération carbonique, carignan.*

Introduction

La vinification par macération carbonique est-elle applicable à l'élaboration des vins rosés?

Des réponses ont été apportées à cette question à l'issue de vinifications conduites dans les conditions de la pratique. Des résultats intéressants ont été obtenus, en particulier, dans les Côtes-du-Rhône et en Côtes-du-Roussillon (BENARD et JOURET, communication personnelle). Cependant, l'un d'entre nous a montré la

nécessité d'entreprendre une expérimentation prolongée avant de proposer une utilisation plus généralisée de cette technique (ANDRÉ, AUBERT et PELISSE, 1971). D'autre part, des études parallèles menées sur l'élaboration de vins blancs par macération carbonique nous avaient montré l'importance de la définition de la température et de la durée d'action de celle-ci au cours de la première phase de fermentation (BENARD *et al.*, 1971).

Aussi, à partir de 1971, avons nous réalisé des essais pour apprécier l'intérêt, sur le plan organoleptique, de la vinification des vins rosés par macération carbonique et pour préciser les conditions optimales de la mise en œuvre de ce procédé.

Méthodes et techniques

Plan général de l'expérimentation

Durant quatre années (1971, 1972, 1974 et 1975) nous avons cherché à mettre en évidence des différences entre les vins élaborés par macération carbonique (MC) et des vins témoins issus de pressurage direct de la vendange (P) ou de saignée (S). Ce dernier système est considéré, souvent, comme le meilleur moyen d'élaboration des vins rosés (ANDRÉ et AUBERT, 1970).

En 1976 et 1977, nous avons voulu préciser les conditions optimales d'utilisation de la MC en vinification de vins rosés de *Carignan*. Le choix du *Carignan* comme matériel expérimental s'explique par le fait que ce cépage est nettement valorisé, pour une vinification en « rouge », par un traitement en MC (travaux en cours); vinifié en vendange foulée, il donne fréquemment des produits assez neutres sur le plan organoleptique (ANDRÉ, AUBERT et PELISSE, 1970*b*).

Matériel végétal

Les essais sont réalisés avec du *Carigan noir* du Domaine expérimental de Pech Rouge (I.N.R.A., Gruissan-Narbonne). Les vendanges sont saines et récoltées à maturité commerciale. L'échantillonnage des lots expérimentaux, de 500 kg environ chacun, a lieu à la vigne selon la technique de BENARD et JOURET (1963).

Protocole expérimental

Les lots destinés à être vinifiés en MC sont déversés sans foulage dans des cuves de 6 hl en acier émaillé. Celles-ci sont placées dans des enceintes thermostatées à 22, 25 ou 35 °C. L'atmosphère des cuves est formée de CO_2 par apport

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Abréviations utilisées dans le texte

MC : vinification par macération carbonique.

S_6, S_{12} : vin obtenu par saignée après foulage de la vendange et macération de 6 ou de 12 h.

P : vin obtenu par pressurage direct de la vendange.

35_{12}, 35_{24}, 35_{36}, 35_{48} : vin élaboré par macération carbonique; la température de l'enceinte où se trouve la cuve est de 35 °C. La durée de la première phase de fermentation est de 12, 24, 36 ou 48 h.

25_{36}, 25_{48}, 25_{72} : vin élaboré par macération carbonique; la température de l'enceinte où se trouve la cuve est de 25 °C. La durée de la première phase de fermentation est de 36, 48 ou 72 h.

22_{60}, 22_{72} : vin élaboré par macération carbonique; la température de l'enceinte où se trouve la cuve est de 22 °C. La durée de la première phase de fermentation est de 60 ou 72 h.

continu de gaz durant tout le temps de cette première phase de vinification. Après des durées variables (12 à 72 h) d'anaérobiose, les lots sont pressurés d'une manière identique avec un pressoir horizontal Vaslin. Le *jus de presse* obtenu est placé en cuve de 6 hl où il subit la fermentation alcoolique puis la fermentation malolactique. La température de la deuxième phase de fermentation (fermentations microbiennes) est maintenue aux alentours de 20-22 °C. Après fermentation malolactique, les vins sont soutirés à l'abri de l'air, sulfités (53 /hl SO_2) puis resoutirés à l'air, sans traitement de collage.

Les lots témoins sont traités soit par pressurage direct soit par foulage-sulfitage (10 /hl SO_2) et saignée après 6 ou 12 h de macération. Les durées de macération avant « saignée » sont relativement longues de façon à favoriser l'extraction des anthocyanes pour des vendanges encuvées à basse température : 16° à 20 °C (Roson, 1967). Le *jus de goutte* obtenu est alors placé après sulfitage (5 /hl SO_2), dans le cas de P dans des conditions de fermentation et de traitements ultérieurs identiques à celles des jus de presse correspondants de MC.

Les vins sont analysés après conservation de 2 à 5 mois en cuve. Les vins de l'expérimentation 1977 ont été embouteillés 3 mois après décuvage et stockés à 15 °C durant 30 mois supplémentaires avant une nouvelle analyse. Les dégustations sont échelonnées sur plusieurs mois.

Analyses

Les mesures de température sont réalisées au niveau des enceintes par thermomètres enregistreurs et dans la masse de vendange au moyen de thermosondes. La température des jus est relevée au décuvage ainsi que, dans certains cas, à différents niveaux de la vendange.

Les analyses physico-chimiques classiques sont effectuées selon les techniques officielles (Journal officiel, 1963). Les résultats de dégustations sont interprétés par le test de Kramer pour une probabilité de 5 p. 100 (Tomassone et Flanzy, 1977).

Les composés phénoliques totaux ont été dosés par la méthode de Folin-Ciocalteu adaptée par Singleton et Rossi (1965). Les anthocyanes totales ont été dosées par les méthodes de Ribereau-Gayon et Stonestreet (1965). Les intensités colorantes rouge (D.O. 520 nm) et jaune (D.O. 420 nm) ont été mesurées en utilisant la méthode de Sudraud (1958). La densité optique à 280 nm a été mesurée en respectant les conditions indiquées par Flanzy et Poux (1958).

Résultats

Résultats globaux

Lors des premiers essais, la maîtrise des températures a été satisfaisante durant les deuxièmes phases de fermentation; il n'en a pas été de même pour les premières phases où les températures des vendanges traitées en macération carbonique ont été faibles, particulièrement en 1971 et 1974.

Les caractéristiques analytiques générales des vins des expérimentations de 1971, 1972, 1974 et 1975 sont regroupées dans le tableau 1.

Les indices relatifs aux substances phénoliques sont donnés dans le tableau 2. Au moment des analyses, la fermentation malolactique n'était terminée que pour certains échantillons.

TABLEAU I

Caractéristiques analytiques des vins

Année = année d'expérimentation. Les analyses sont effectuées (×) mois après le début de l'expérimentation.
Lot = les sigles représentatifs de chaque vin sont commentés à la première page du mémoire.
Acidité totale = le signe + ou — entre parenthèses signifie que la fermentation malolactique était effectuée ou pas au moment de l'analyse.

Année	Lot	d_{20}^{20}	GL 20°	Extrait sec (g /l)	Sucres (g /2)	Glycérol (g /l)	Aldéhydes (mg /l)	Azote total (mg /l)	Acidité totale H_2SO_3 (g /l)	Acidité volatile H_2SO_3 (g /l)	pH	Alcalinité cendres (meq /l)
1971 (3)	35_{48}	0,9933	11 53	19,25	1,51	6,80	28	84	4,60 (—)	0,16	3,41	30
	22_{72}	0,9923	11 48	15,98	1,35	8,09	18	77	3,50 (+)	0,18	3,49	25
	S_6	0,9917	11 54	13,38	1,58	6,53	19	84	2,90 (+)	0,24	3,56	24,5
	S_{12}	0,9928	11 48	17,00	1,69	6,62	37	105	3,70 (—)	0,20	3,48	27
	P	0,9918	11 43	14,60	1,28	7,26	18	105	2,90 (+)	0,31	3,55	24
1972 (2)	35_{36}	0,9918	11 85	15,45	1,25	6,99	18	84	4,50 (+)	0,24	3,42	27
	25_{72}	0,9928	11 85	18,10	1,60	7,17	35	84	5,40 (—)	0,24	3,42	30
	S_{12}	0,9930	11 67	20,25	2,95	7,17	40	112	6,00 (—)	0,30	3,30	27,5
	P	0,9923	12 09	19,25	1,10	7,36	28	105	5,90 (—)	0,22	3,33	28,5
1974 (4)	35_{36}	0,9928	11 38	20,30	1,92	4,14	40		3,50 (+)	0,19	3,38	23
	25_{72}	0,9939	11 05	21,90	1,29	3,68	49	112	3,70 (+)	0,20	3,40	23,5
	S_{12}	0,9938	11 33	22,70	3,68	3,95	48	133	3,80 (—)	0,31	3,37	23
1975 (5)	35_{12}	0,9929	10 57	18,30	1,94	8,18	40	56	3,80 (+)	0,32	3,25	18,5
	35_{36}	0,9939	10 19	19,30	2,22	9,01	44	84	3,90 (+)	0,36	3,25	20
	25_{48}	0,9949	10 52	22,90	4,72	7,99	16	56	2,90 (+)	0,27	3,68	26,5
	22_{60}	0,9935	10 57	19,60	2,24	9,20	42	77	3,40 (+)	0,30	3,32	19,5
	S_{12}	0,9923	10 62	16,70	1,00	7,45	51	91	3,90 (+)	0,26	3,26	18

TABLEAU 2

Caractéristiques chromatiques des vins

Année = année d'expérimentation. Les analyses sont effectuées (×) mois après le début de l'expérimentation.
Lot = les sigles représentatifs de chaque vin sont commentés à la première page du mémoire.

Année	Lot	DO 520.10³ nm	DO 420.10³ nm	DO 280.10³ nm	$\frac{DO\ 520}{DO\ 420}$	$\Sigma \frac{DO\ 520}{DO\ 420}$ .10³	$\frac{DO\ 520}{DO\ 280}$
1971 (3)	35_{48}	146	48	395	3,04	194	0,36
	22_{72}	131	44	375	2,97	175	0,34
	S_6	43	27	224	1,59	70	0,19
	S_{12}	76	36	285	2,11	112	0,26
	P	46	26	228	1,76	72	0,20
1972 (2)	35_{36}	83	28	258	2,96	111	0,32
	25_{72}	70	28	284	2,50	98	0,24
	S_{12}	45	18	212	2,50	63	0,21
	P	56	24	218	2,33	80	0,25
1974 (4)	35_{36}	109	38	292	2,86	147	0,37
	25_{72}	124	42	302	2,95	166	0,41
	S_{12}	100	32	264	3,12	132	0,37
1975 (5)	35_{12}	47	20	176	2,35	67	0,26
	35_{36}	89	30	240	2,96	119	0,37
	25_{28}	230	70	570	3,28	300	0,40
	22_{60}	70	20	232	3,50	90	0,30
	S_{12}	38	20	175	1,90	58	0,21

TABLEAU 3

Dégustation des vins

L'interprétation des résultats des jurys de dégustation (essai de préférence) se fait au moyen du test de Kramer (TOMASSONE et FLANZY, 1977).

Année = année d'expérimentation.
Date = nombre de mois après le début de l'expérimentation.
n = nombre de dégustateurs.
K = limites de la table de Kramer au seuil 5 p. 100. Le couple supérieur de valeurs indique si une différence significative existe entre les vins. Dans l'affirmative (H_1), le couple inférieur de valeurs permet de préciser cette différence.

Classement des échantillons = les vins sont rangés (ligne supérieure) par ordre croissant des sommes de rangs obtenus (ligne inférieure). Les sommes sortant des limites de K correspondent à des vins jugés supérieurs (valeurs plus basse que la limite inférieure de K) ou inférieurs (valeur plus élevée que la limite supérieure de K) aux autres.

H = H_0 : pas de différences significatives entre vins;
H_1 : différences significatives entre vins.

Année	Date	*n*	K	Classement des échantillons somme des rangs					H
1971. . .	2	12	25-47 28-44	S_{12} 25	35_{48} 33,5	P 36	22_{72} 37	S_6 47	Limite H_0
	7	13	27-51 31-47	22_{72} 26	S_{12} 31	35_{48} 39	S_6 42,5	P 56,5	H_1
1972. . .	3	12	21-39 24-36	S_{12} 23,5	35_{36} 27,5	25_{72} 33	P 36		H_0
	3	9	13-23 14-22	$[(\frac{1}{2})$ P + $(\frac{1}{2})$ $35_{36}]$ 14		$[(\frac{1}{2})$ P + $(\frac{1}{2})25_{72}]$ 19	S_{12} 21		H_0
	7	16	30-50 33-47	35_{36} 27	25_{72} 33,5	S_{12} 43,5	P 56		H_1
1974. . .	8	8	11-21 12-20	25_{72} 14	S_{12} 35_{36} 17				H_0
	21	4	— —	35_{36} 4	25_{72} 8				—
1975. . .	9	4	6-18 7-17	35_{36} 5	35_{12} 22_{60} 10	S_{12} 17	25_{48} 28		H_1
1977. . .	33	12	25-47 28-44	35_{12} 27	25_{36} 32	S_{12} 34	35_{24} 35	35_{36} 52	H_1

Les vins de pressurage direct (P) ou de saignée (S) ont des concentrations en azote plus élevées que celles des vins correspondants de macération carbonique (MC). Inversement, les indices de substances phénoliques sont les plus faibles dans les vins P et S.

La température et la durée de macération influencent fortement la composition des lots MC. Ainsi 72 h de stockage de la vendange, en anaérobiose, à 25 °C augmentent davantage la teneur en extrait sec, en alcalinité des cendres et, généralement, en polyphénols qu'un traitement de 36 h à 35 °C ou de 72 h à 22 °C.

Les résultats de dégustation sont schématisés dans le tableau 3. Pour les deux essais de 1971 et 1972, les vins P sont souvent jugés inférieurs aux autres échantillons. Les vins S sont d'autant plus appréciés que la durée de macération avant saignée a été longue (12 h).

La macération carbonique apporte des caractères odorants et aromatiques considérés comme fins et agréables par l'ensemble des dégustateurs. Par contre la coloration des vins est fréquemment jugée comme trop importante et la saveur bien que fruitée, trop tannique. Les produits obtenus par cette technique présentent des caractéristiques visuelles et gustatives plus proches de celles de « vins de café » que de celles de vins rosés.

Résultats relatifs à la recherche des conditions optimales de MC

Un ensemble de résultats relatifs à l'expérimentation 1977 est présenté dans le tableau 4.

Les températures obtenues au sein de la vendange en fin de macération sont proches de celles souhaitées.

Au cours de cette phase, les sucres et l'acide malique sont partiellement transformés. Nous postulons que, après 12 h de macération, la concentration en malate du lot S_{12}, pris comme référence, est égale à celle de la vendange initiale. Dans ces conditions, le pourcentage de malate catabolisé croît régulièrement avec la durée de stockage dans les lots de macération carbonique placés à 35 °C. Parallèlement, la production d'éthanol augmente brusquement dans les lots 35_{36}. Pour ces deux critères, le lot 25_{36} se situe entre les lots 35_{36} et 35_{24}.

La fermentation malolactique est terminée un mois après le début de l'expérimentation pour les quatre échantillons de macération carbonique alors qu'elle n'a pas encore débutée pour S_{12}. Cette fermentation est achevée dans tous les vins au troisième mois de stockage.

Tant au décuvage qu'après trois mois de conservation, le rangement des vins par valeurs décroissantes pour l'ensemble des critères de substances phénoliques (à l'exception de D.O. 420 nm) donne le classement suivant :

$$35_{36} > 25_{36} \geqslant S_{12} > 35_{24} > 35_{12}$$

Entre les décuvages et le troisième mois de stockage, le rapport DO 520/DO 420 augmente. Les coefficients de variation sont respectivement de :

$$S_{12} = 2{,}13;\ 35_{12} = 1{,}66;\ 35_{24} = 1{,}19;\ 35_{36} = 1{,}07;\ 25_{36} = 1{,}07$$

Dans la même période, le rapport DO 520/DO 280 diminue avec des coefficients d'évolution de :

$$35_{36} = 0{,}89;\ 25_{36} = 0{,}86;\ S_{12} = 0{,}77;\ 35_{24} = 0{,}75$$

TABLEAU 4

Expérimentation 1977. Composition des jus et des vins

Caractéristiques des échantillons.

Les jus sont analysés au décuvage après macération. Les températures des vendanges aux niveaux supérieurs et inférieurs des cuves ont été mesurées

	S_{12}	35_{12}	35_{24}	35_{36}	25_{36}
niveau supérieur	24	29,5	35	37,5	22
niveau inférieur	27	30	35	37,5	24

Les vins sont analysés 3 mois après décuvage, les fermentations alcooliques et malolactiques étant terminées. Ils sont à nouveau analysés et dégustés après embouteillage et 30 mois supplémentaires de stockage.

Conventions.

M catabolisé p. 100 = pourcentage de malate catabolisé en prenant pour valeur de départ la concentration retrouvée dans S_{12} au décuvage.
Polyphénols (g/l) = teneur en polyphénols exprimée en grammes d'acide gallique par litre.
Anthocyanes (g/l) = teneur en anthocyanes exprimée en grammes par litre.

Date des analyses	Décuvage					3 mois après décuvage					33 mois après décuvage				
Échantillon	S_{12}	35_{12}	35_{24}	35_{36}	25_{36}	S_{12}	35_{12}	35_{24}	35_{36}	25_{36}	S_{12}	35_{12}	35_{24}	35_{36}	25_{36}
Sucres (g/l)	185	183,5	179,5	173	181,5	1,5	1,4	1,4	1,5	1,4					
°GL	0°12	0°25	0°37	0°89	0°57	11°14	11°44	11°00	11°33	11°00	11°09	11°33	10°87	11°24	11°24
M = acide malique (mg/l)	61	57,5	48,5	38	46	0	0	0	0	0	0	0	0	0	0
M catabolisé p. 100	0	6	21	38	24	100	100	100	100	100	100	100	100	100	100
D.O. 520 nm × 10^3	145	82	126	184	133	88	64	82	135	105	32	24	27	47	35
D.O. 420 nm × 10^3	102	64	55	61	46	30	30	30	42	34	18	15	18	30	22
D.O. 280 nm × 10^3	320	\	285	385	297	260	225	242	320	270	237	205	210	273	233
D.O. 520 + D.O. 420 10^3	242	146	181	245	179	118	94	112	177	139	50	39	45	77	57
D.O. 520/D.O. 420	1,37	1,28	2,29	3,01	2,89	2,93	2,13	2,73	3,21	3,08	1,78	1,60	1,50	1,56	1,59
D.O. 520/D.O. 280	0,43	\	0,44	0,47	0,44	0,33	0,28	0,33	0,12	0,38	0,14	0,12	0,13	0,17	0,11
Polyphénols totaux (g/l) × 10^3	524	467	600	810	630	513	399	444	592	501	513	410	467	615	513
Anthocyanes (g/l) × 10^3	157	91	150	239	175	\	\	114	193	175	30	22	21	35	33

Le rapport DO 520/DO 280 continue à décroître pour tous les vins, après 33 mois de conservation. Les coefficients de variation sont respectivement de :

$$S_{12} = 0{,}42;\ 35_{12} = 0{,}43;\ 35_{24} = 0{,}39;\ 35_{36} = 0{,}40;\ 25_{36} = 0{,}30.$$

Parallèlement, le rapport DO 520/DO 420 diminue. Il conserve, cependant, des valeurs supérieures à celles du départ pour les deux lots S_{12} et 35_{12}. Ces échantillons ont donc une nuance plus rouge qu'au décuvage, tout en ayant un rapport DO 520/DO 280 apparemment plus favorable (AUBERT, communication personnelle) que celui du départ.

L'interprétation statistique des résultats de la dégustation des lots au trente troisième mois de stockage (tabl. 3) fait ressortir deux vins : 35_{12} est jugé comme significativement le meilleur alors que 35_{36} est considéré comme le moins bon.

Discussion

Le passage par un stockage préalable de la vendange de Carignan non foulée dans une atmosphère de gaz carbonique conduit à l'obtention de vins rosés plus typés aromatiquement et gustativement que les vins témoins de pressurage direct. Cette observation se vérifie parfois avec les témoins issus d'une vinification par saignée de 12 h.

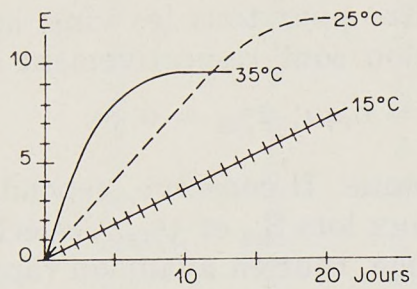
Cependant, la teneur en substances phénoliques des lots MC apparaît souvent trop élevée pour un classement des produits en catégorie « vins rosés ». A la dégustation l'assemblage de ces produits organoleptiquement très marqués avec des vins P ou S tend à être préféré à chacun d'entre eux pris séparément (tabl. 3, 1972). L'importance de la coloration des vins rosés sur leur acceptation finale par le dégustateur a été signalée (ANDRÉ, AUBERT et PELISSE, 1970 *a* ; ROSON et JOURET, 1975) de même que l'intérêt présenté par les assemblages des vins (ANDRÉ, AUBERT et PELISSE, 1970 *b*).

Lors de l'élaboration des vins rosés, il convient de porter une attention spéciale à trois phénomènes : l'extraction ou la formation de substances aromatiques; la diffusion des substances phénoliques; l'atteinte d'une acidité correcte.

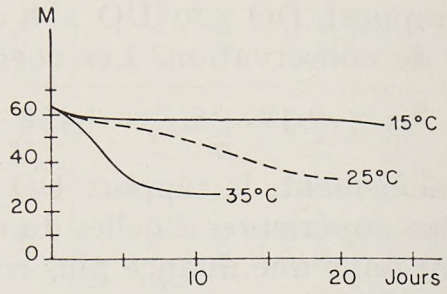
L'utilisation judicieuse de chacun d'eux devrait permettre d'obtenir des vins aromatiques, sapides, suffisamment acides, et d'un degré de coloration correct. Il est cependant difficile de dissocier totalement ces phénomènes; le praticien est souvent conduit, soit à un compromis, soit à favoriser systématiquement l'un des composants du produit final au détriment des autres.

Dans nos essais, la fermentation malolactique ayant été recherchée, l'équilibre acide des vins s'est effectué sans intervention de notre part. En revanche, la flaveur, la coloration et la tanicité des vins ont été directement liées au choix des conditions de température et de durée d'action du métabolisme anaérobie. Par exemple, 72 h de macération à 25 °C donnent des vins s'éloignant davantage des caractéristiques d'un « rosé » que les vins correspondants issus d'une macération de 36 h à 35 °C : les premiers présentent davantage de substances phénoliques pour une intensité aromatique peu différente.

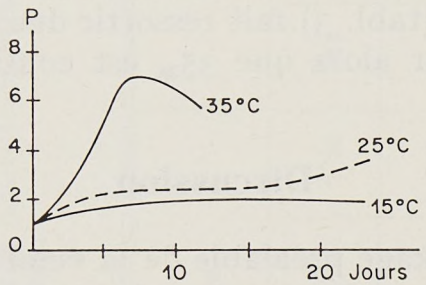
Ce résultat est prévisible à partir des diagrammes de formation d'éthanol et de diffusion des polyphénols (fig. 1; FLANZY et ANDRÉ, 1973). En effet, la cinétique de formation d'éthanol est un bon marqueur de l'allure de ce métabolisme et, par extension, un indice des qualités organoleptiques du produit final. D'autre part, la charge des vins en polyphénols est fonction de l'intensité du passage de ces substances des cellules de la pellicule aux cellules de la pulpe durant la macération.



Température et production d'éthanol
E = éthanol en g pour 1 000 g de baies



Température et disparition de l'acide malique
M = acide malique en mEq/l



Température et diffusion des polyphénols
P = polyphénols en mg/l d'acide gallique

FIG. 1. — *Évolution de différents substrats au cours du métabolisme anaerobie de la baie de raisin en fonction de la température*

Schémas selon FLANZY et ANDRÉ (1973).

Jusqu'à 36 h environ de macération, les extractions de substances polyphénoliques sont sensiblement identiques à 25 et 35 °C alors que la teneur en alcool à 35 °C est la plus élevée. Pour obtenir une valeur identique de ce dernier paramètre, la durée de macération devra être d'autant plus longue que la température sera plus basse : 4 j à 25 °C, 8 j à 15 °C, 36 h à 35 °C.

L'évolution des rapports DO 520/DO 420 et DO 520/DO 280 durant les fermentations microbiennes (levures, bactéries malolactiques) et la conservation de 3 mois (tabl. 4) semblent montrer que les vins 35_{36} et 25_{36} tendent à prendre une teinte plus jaune tout en conservant un meilleur équilibre anthocyanes-tanins que les autres lots. Ceci avait déjà été noté (ANDRÉ, 1975). A partir d'une certaine température (25 °C), la durée de son action modifierait sensiblement le type ou l'évolution des substances extraites.

L'étude théorique et les résultats expérimentaux conduisent donc à conseiller l'application d'une température élevée durant un temps bref. Cette formule permet en outre de limiter le temps d'occupation de la cuverie au cours de la première phase et de réduire la durée totale de vinification. Cependant, dans le cas des conditions thermiques défavorables (vendanges froides à la récolte) il sera nécessaire de prolonger la phase de macération.

L'atteinte rapide des températures élevées dans une masse de vendange entière est souvent difficile (FLANZY *et al.*, 1980). En 1977, après 12 h de macération, la masse de la vendange était à 30 °C; elle passait à 35 °C après 24 heures et atteignait 37 °C à la trente sixième heure.

Compte tenu des moyens limités dont dispose le praticien pour maîtriser ce facteur, la température des baies à l'encuvage prend une grande importance dans la réussite de la vinification. Ainsi, durant 3 années (1971, 1974, 1976) sur les 6 lots d'expérimentation, les températures des lots en MC ont été inférieures de 10 à 15 °C à celle recherchée (35 °C) et effectivement obtenue dans les enceintes conte-

nant les cuves; les vendanges avaient été cueillies aux environs de 17-18 °C.

Inversement, une température trop élevée peut amener une diminution de la qualité des polyphénols extraits (tabl. 4; lot 35_{36}).

Le tableau 4 permet de noter la part vraisemblablement importante prise par le métabolisme anaérobie dans le catabolisme du malate durant les quelques heures de la première phase de MC.

De même que pour la production d'éthanol, les valeurs obtenues sont supérieures à celles généralement observées dans les expérimentations de laboratoire sur le métabolisme anaérobie strict (Flanzy, 1978). Ce fait a déjà été signalé (Flanzy et André, 1973) sans qu'une interprétation pleinement satisfaisante soit actuellement avancée.

Quoiqu'il en soit, l'accélération constatée des phénomènes métaboliques peut expliquer l'importance prise par cette brève phase de la vinification sur les caractéristiques organoleptiques des vins.

Conclusions

La vinification par macération carbonique peut permettre d'élaborer des vins rosés de Carignan fruités et fins. Ces vins sont obtenus après fermentations microbiennes des *jus de presse* issus de vendange stockée dans une cuve dont l'atmosphère est formée de gaz carbonique.

Afin d'éviter une trop grande richesse du produit final en substances phénoliques, il importe de jouer dans la première phase de fermentation sur le couple de facteurs : température et durée de stockage.

Théoriquement, il paraît souhaitable d'obtenir une haute température de la vendange durant un temps réduit. Dans la mesure où ces conditions sont réalisables pratiquement, une macération de 24 à 36 h et une température de la masse de vendange de 30 °C doivent correspondre à la formule optimale.

Si l'obtention rapide d'une telle température n'est pas possible, il est nécessaire d'effectuer une macération vers 20-22 °C durant 3 à 5 j.

Les valeurs proposées doivent être ajustées, évidemment, en fonction du type de vin rosé recherché. De même la vinification en rosé d'autres cépages, effectuée avec succès par certains praticiens, peut exiger des conditions de réalisation assez différentes.

Dans nos essais, les vins de macération carbonique sont classés par les dégustateurs à un niveau voisin de celui des vins de saignée de 12 h et à un niveau nettement supérieur à celui des vins de pressurage direct.

Élaborés dans de bonnes conditions (température-durée de macération) les vins de macération carbonique peuvent être consommés tels quels ou servir d'élément améliorateur dans des assemblages.

Certains équipements faciliteraient vraisemblablement la mise en œuvre de la macération carbonique pour l'élaboration de vins rosés et de vins blancs. Ainsi, il pourrait être intéressant de réaliser la première phase de fermentation dans une chambre où la vendange serait stockée, éventuellement, dans les récipients de ramassage. La composition de l'atmosphère de cette chambre, sa température, son hygrométrie devraient être régulées.

La maîtrise des différents phénomènes impliqués dans le métabolisme anaérobie pourrait être ainsi améliorée; ceci conduirait à un « pilotage » plus précis dans l'élaboration de ces types de vins.

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Symmary

Carbonic maceration of wine making. Elaboration of pink wines

Carbonic maceration winemaking was utilized for elaboration of pink wines of *Carignan*. The wines get by this technic are judged for testing to be better than those obtained by direct pressing of grapes; they are at a nearly level of wines obtained by short maceration.

Still, a precise control of temperature and duration of the first period of fermentation is necessary to obtain enough aromatic products with a low level of phenolic substances.

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Fermentation méthanique avec séparation des phases acidogène et méthanogène appliquée au traitement des effluents à forte charge polluante (distilleries)

A. BORIES

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Résumé

La fermentation méthanique en deux phases séparées (acidogénèse et production de méthane), est étudiée sur une eau résiduaire de distillerie (vinasse de vin) à très forte charge polluante (DCO : 85 000 mg O_2 /l).

L'étude des deux phases met en évidence le métabolisme intermédiaire des acides gras volatils. Ce procédé se révèle comme un moyen de contrôle et de conduite de la fermentation.

En phase acidogène une forte production d'AGV est observée (AVT : 580 mÉq /l, 35 g /l en acide acétique). Les acides butyrique (35-40 p. 100), propionique (30-35 p. 100) et acétique (15-25 p. 100) sont les produits majeurs obtenus à partir des principaux composés de la vinasse : glycérol et acide lactique. La cinétique acidogène et le bilan de la conversion en AGVsont examinés.

En phase méthanogène, les populations microbiennes adaptées à ce profil d'AGV, assurent la dégradation complète des acides, ainsi que des substances secondaires. Aucune inhibition de la production de méthane par l'acide propionique n'est observée, même à forte concentration (168 mM /l).

Les performances en épuration sont très élevées (98 p. 100). Les paramètres cinétiques : temps de séjour hydraulique, charge appliquée, temps de rétention des solides, productivité en méthane, sont déterminés sur fermenteurs infiniment mélangés.

Le bilan énergétique de la fermentation méthanique des vinasses est comparé à celui de l'autre voie d'épuration par oxydation biologique. De plus, l'analyse du bilan de la distillation montre que la potentialité énergétique de la fermentation peut représenter 40 p. 100 de l'énergie consommée à la distillation d'alcool.

Mots clés : *méthane, acides gras volatils, séparation des phases, épuration, eaux résiduaires, distilleries, énergie.*

Introduction

La bioconversion des matières organiques par fermentation méthanique s'adresse à un vaste domaine : biomasses humides (terrestres, aquatiques) sous produits agricoles, déchets et eaux résiduaires. C'est dans le secteur du traitement des effluents que son intérêt immédiat le plus marqué se manifeste par le couplage épuration-production d'énergie. Pour des rejets à forte charge polluante, la compétitivité du procédé anaérobie apparaît nettement vis-à-vis des autres systèmes d'épuration, consommateurs d'énergie, dont le coût grandissant compromet leur utilisation.

Si les perspectives énergétique et économique sont très séduisantes, la faisabilité technologique de la fermentation méthanique n'est pas complètement acquise. La fiabilité, la maîtrise, critères de valeur d'un traitement d'épuration, demandent à être développées.

Les récents travaux sur la microbiologie et la biochimie de la digestion anaérobie apportent des connaissances décisives sur les mécanismes (Taylor, 1976; Wolin, 1976; Bryant, 1976; Wolfe., 1979; Zeikus, 1979). Mais ils soulignent aussi leur complexité. Les moyens de contrôle et d'intervention demeurent limités.

Le métabolisme intermédiaire des acides gras volatils (AGV) joue un rôle déterminant dans le déroulement du processus. L'équilibre fragile entre la synthèse des AGV et leur dégradation conditionne la stabilité de la fermentation. Dans un but d'optimisation cinétique, la séparation entre la phase production d'AGV et la phase de production de méthane a été proposée.

Ces travaux ont essentiellement porté, soit sur la digestion anaérobie de matières solides : Ghosh *et al.*, 1975; Keenan, 1976, soit sur la fermentation de milieux synthétiques à faible charge organique (Pohland et Massey, 1975; Massey et Pohland, 1978; Ghosh et Klass, 1978; Couplet *et al.*, 1979; Cohen *et al.*, 1979).

Paradoxalement, la fermentation en deux phases sur les effluents à très forte charge polluante n'a été que très peu étudiée (Bories et Maugenet, 1979 *a.*). C'est pourtant dans le cas de tels milieux que les risques de déséquilibre en fermentation monoétage sont les plus grands. La séparation des phases apparaît comme un moyen de contrôle.

Les objectifs de notre étude sont d'examiner le comportement (métabolisme, cinétique) de chaque phase et les conséquences de leur séparation en fermentation d'eaux résiduaires de distillerie très concentrées en matières organiques solubles (DCO : 85 000 mg O_2/l). La faisabilité de ce traitement d'épuration est étudiée par la détermination des performances et des paramètres de conduite des fermentations. Le bilan énergétique et la potentialité de la fermentation méthanique des vinasses sont analysés comparativement à l'autre voie d'épuration (oxydation biologique) et dans le contexte de la distillation d'alcool.

Matériels et méthodes

Matière première : vinasse de vin

L'effluent retenu dans cette étude provient de la distillation d'eau de vie de vin blanc (Cognac). La vinasse (phase désalcoolisée du vin) subit industriellement une préconcentration thermique, d'environ 3 fois, préalable à la récupération

d'acide tartrique effectuée par précipitation à la chaux à pH 4,7. La vinasse préconcentrée, détartrée, constitue le milieu utilisé dans nos expérimentations (tabl. 1).

Matériels de fermentation

Les fermentations sont conduites en réacteurs anaérobies infiniment mélangés, maintenus à 35 °C. Leur capacité utile est de 2 l, 6 l, et 20 l. L'équipement périphérique de chaque fermenteur consiste en :

— un compteur à gaz volumétrique (Schlumberger), mesurant les productions de gaz;

— une poche souple, pour recueillir les échantillons de gaz de fermentation destinés à l'analyse. Ce dispositif permet aussi de conserver intacte l'enceinte gazeuse du fermenteur lors du prélèvement du milieu de fermentation;

— une pompe péristaltique double canal, assurant l'apport du substrat et le soutirage du milieu fermenté pour les fermentations continues.

La conduite en ligne de la fermentation en deux phases met en jeu deux fermenteurs montés en série. Le premier, de 2 l ou 6 l, est utilisé pour la phase acidogène, le second (20 l), pour la phase méthanogène. Un stockage intermédiaire du milieu fermenté est pratiqué pour harmoniser les débits entre les deux étages.

Dans certains essais, un vase de décantation (1 l) est associé au deuxième étage afin de recirculer les boues méthanogènes et modifier leur temps de rétention.

Origine et obtention des flores microbiennes

L'origine des populations microbiennes sélectionnées en fermentation de vinasses est la flore de digesteurs anaérobies de boues d'épuration d'effluents urbains (ville de Narbonne). Une double sélection est opérée pour l'obtention des phases acidogène et méthanogène.

L'implantation de populations acidogènes a été obtenue par inoculation de boues de digesteurs sur vinasse de vin blanc neutralisée et à 35 °C. La flore acidogène sélectionnée est conservée par transfert successif sur vinasse.

L'adaptation de populations méthanogènes est réalisée dans un deuxième temps par enrichissement progressif d'un échantillon de digesteur de boues d'épuration (20 l) par la vinasse préalablement fermentée en phase acidogène. La flore méthanogène sélectionnée est conservée d'essai en essai.

Les deux types de flores ont été maintenus sans problème sur plus de trois années.

Méthodes analytiques

— Acidité volatile totale (AVT) : l'échantillon (de 5 à 20 ml), acidifié par l'acide sulfurique jusqu'à pH 1 puis décarboniqué sous vide, est entraîné par la vapeur d'eau à l'appareil de Jaulmes. Le distillat (250 ml) est titré par la soude 0,1 N. Les résultats sont exprimés, soit en milliéquivalent de soude /l (méq /l) soit en gramme d'acide acétique /l. Bien que cette dernière expression soit très largement utilisée, son interprétation apparaît confuse quand les acides dominants sont autres que l'acide acétique.

— Acides gras volatils (AGV). L'analyse des AGV est effectuée à partir du distillat obtenu dans les mêmes conditions que précédemment. Après neutralisation (pH 9-10) puis concentration sous vide (55 °C), les acides gras salifiés sont repris par une solution d'acide sulfurique en excès. Les conditions de l'analyse par chromatographie en phase gazeuse sont : colonne en verre, longueur 1,6 m, diamètre 6,3 mm, support chromosorb W-AW 80-100 mesh; phase SP 1 200 (10 p. 100) et acide phosphorique (1 p. 100); température isotherme 125 °C; gaz vecteur : azote 44 ml/mn; détecteur : ionisation de flamme.

— Gaz de fermentation : dosage du gaz carbonique et du méthane par chromatographie en phase gazeuse sur colonne de silicagel (longueur 0,5 m, diamètre 3,2 mm, granulométrie 80-100 mesh) et tamis moléculaire 5 X (longueur 2 m, diamètre 3,2 mm).

Température 40 °C, gaz vecteur hélium, détecteur à conductibilité thermique. Résultats exprimés en p. 100.

— Carbone organique total (COT) : analyseur DOHRMANN DC 50 (Bories, 1978).

— Demande chimique en oxygène (DCO) : norme AFNOR T 90.111.

— Demande biochimique en oxygène (DBO) : norme AFNOR T 90.103.

— Glycérol, acide lactique, acide malique : méthodes enzymatiques en flux continu (Batlle *et al.*, 1978; Batlle et Collon, 1979).

— Azote total : méthode selon Kjeldahl. Résultats en mg N/l.

— Azote ammoniacal : distillation par entraînement à la vapeur d'eau, à pH 7,4 en présence de tampon phosphate (Standard Methods, 1971, 13th edition, p. 45). Le distillat, recueilli dans une solution saturée d'acide borique en présence de rouge de méthyle et vert de bromocrésol, est titré par l'acide chlorhydrique 0,02 N. Les résultats sont exprimés en mg N (NH_4)/l.

— Alcalinité totale : méthode potentiométrique (Standard Methods, 13th Edition, 1971, p. 55). La correction due à l'interférence de l'acidité volatile totale est effectuée selon les indications de Brovko *et al.*, 1977. Résultats exprimés en g $CaCO_3$/l.

— Matières en suspension (MES) : pesée du culot de centrifugation après séchage à 105 °C.

— Cations : calcium, potassium, sodium : spectrophotométrie de flamme.

Résultats

Phase acidogène

1. — *Évolution des constituants et formation des AGV.*

L'analyse des vinasses de vin blanc mentionnée dans le tableau 1 fait apparaître une forte charge polluante, représentée par des matières organiques solubles. Le glycérol et l'acide lactique sont les deux composés majeurs de cet effluent. Les nombreuses autres substances qui entrent à faibles concentrations dans la constitution des vins sont également présentes dans ces vinasses (Puisais, 1975).

TABLEAU I

Composition type des vinasses de vins blancs préconcentrées et détartrées

pH	4,7
MES g/l	2-5
COT mg C/l	30 000-36 000
DCO mg O_2/l	78 000-85 000
DBO mg O_2/l	37 000-41 000
Glycérol g/l	18,6-25,8
Acide lactique g/l	13-21.0
Acide malique g/l	1- 9,5
Azote total mg N/l	1 400- 1 500
Phosphore total mg P/l	300- 450
Potassium mg/l	4 750- 5 150
Sodium mg/l	98- 166
Calcium mg/l	3 800- 4 700

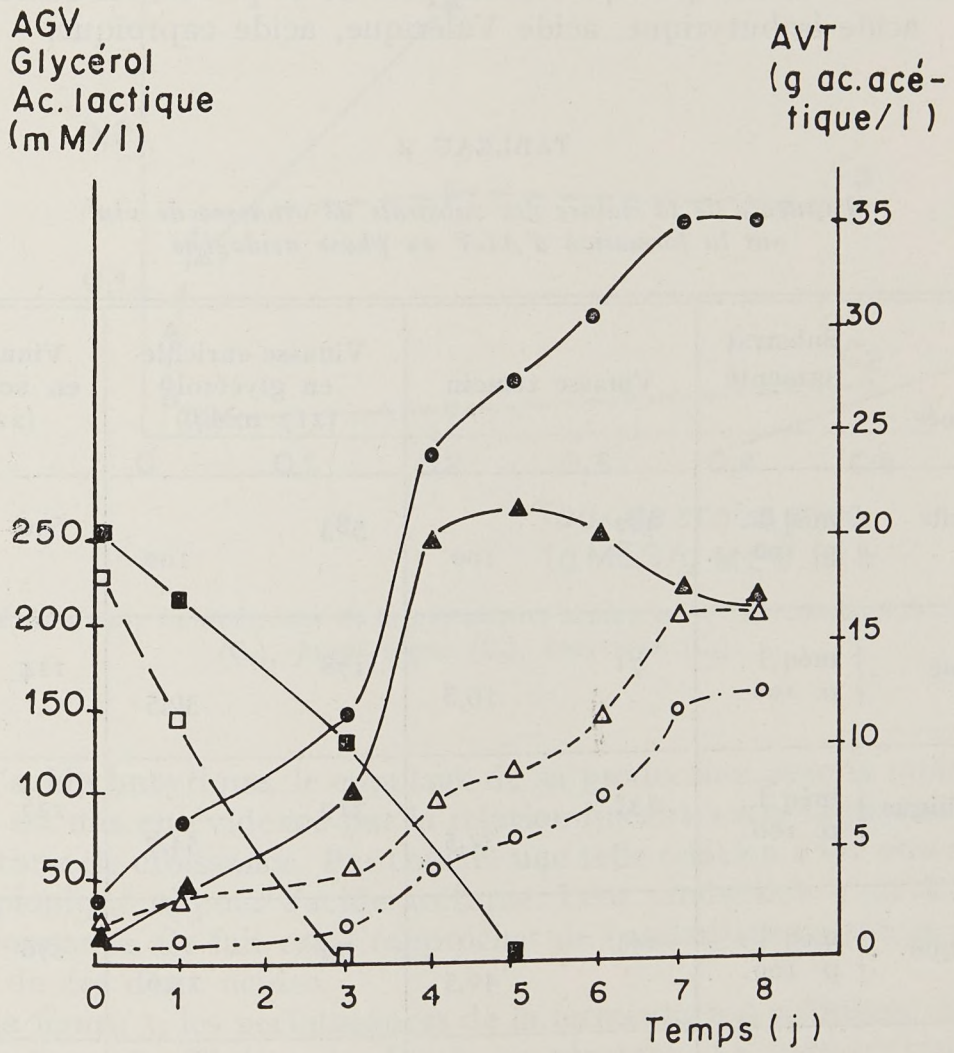

FIG. 1. — *Évolution des substrats :* glycérol (□— — —□), acide lactique (■ ——— ■) et production des acides acétique (o —.— o), propionique (△ ---------- △), butyique (▲ ——— ▲) et de l'AVT (● ——— ●) *au cours de la phase de fermentation acidogène de vinasse de vin, préconcentrée et détartrée.*

L'acidité des vinasses est corrigée avant fermentation par neutralisation jusqu'à pH 7, soit par la soude, soit par la potasse.

Inoculée à raison de 5 p. 100 par la flore acidogène sélectionnée, la vinasse est le siège d'une intense production d'AGV (AVT : 580 méq /l), représentés principalement par les acides butyrique (40 p. 100), propionique (34 p. 100) et acétique (25 p. 100) (fig. 1). La formation de l'acide butyrique est la plus rapide et se superpose à la phase de croissance exponentielle de la population bactérienne. Les acides acétique et propionique voient leur production étalée sur une plus longue durée; elle se poursuit même bien après l'épuisement du milieu en ses deux composés majeurs (glycérol, acide lactique). Le glycérol est consommé dès le début de la fermentation, avant même qu'une production d'acide soit observée.

L'enrichissement du milieu de fermentation en chacun des deux substrats souligne l'origine des AGV (tabl. 2). En présence de glycérol, l'acide propionique est le produit majeur de la fermentation (55 p. 100 des AGV). La synthèse d'acide acétique est également accrue (30,5 p. 100). Par contre, la production d'acide butyrique est diminuée de moitié.

L'apport d'acide lactique (224 m M /l) ne modifie par les proportions des acides par rapport à l'essai témoin. Cependant les concentrations en acides butyrique et propionique sont quantitativement augmentées de 105 mM /l et 90 mM /l respectivement.

Outre ces trois acides qui représentent près de 90 p. 100, les autres AGV produits sont : acide isobutyrique, acide Valérique, acide caproïque.

TABLEAU 2

Influence de la nature des substrats de vinasses de vin sur la formation d'AGV en phase acidogène

Quantité d'AGV formée / Substrat fermenté		Vinasse témoin	Vinasse enrichie en glycérol (217 mM /l)	Vinasse enrichie en acide lactique (224 mM /l)
Acidité volatile totale	méq /l	422	583	650
	p. 100	100	100	100
Acide acétique	méq /l	71	178	114
	p. 100	16,8	30,5	17,5
Acide propionique	méq /l	137	319	227
	p. 100	32,5	54,7	34,9
Acide butyrique	méq /l	171	79	276
	p. 100	40,5	13,6	42,5
$C_2 + C_3 + C_4$	méq /l	379	576	617
	p. 100	89,8	98,8	94,9

2. — *Cinétique de l'acidogénèse*

Les évolutions différentes notées dans la formation des acides (fig. 1) suggèrent l'existence de plusieurs types de relations entre la croissance bactérienne et la production en AGV. Dans la figure 2, la productivité spécifique des AGV (vitesse de production par unité de biomasse) est examinée en fonction du taux de croissance de la population acidogène.

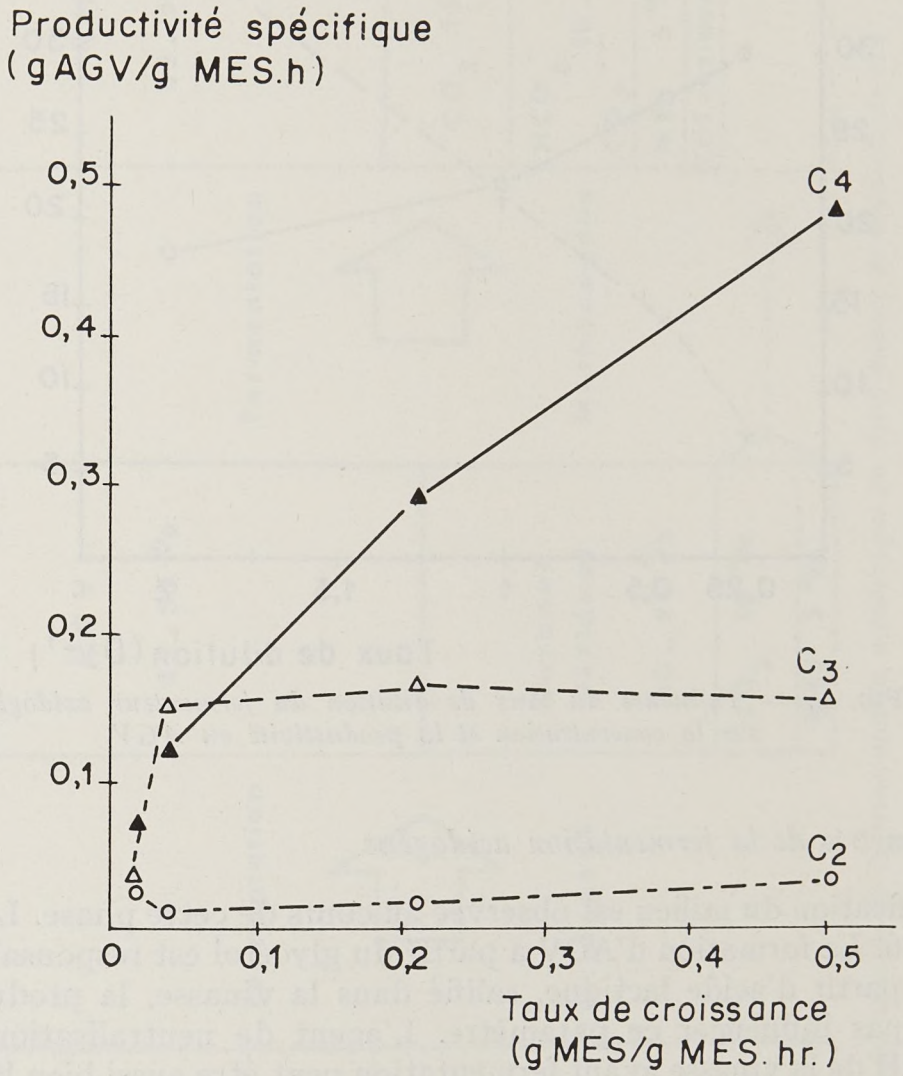

FIG. 2. — *Relation entre la croissance de la population acidogène et la production des acides acétique* (C_2), *propionique* (C_3), *butyrique* (C_4).

Pour l'acide butyrique, le couplage de sa production avec la croissance de la population est mis en évidence par la relation linéaire entre la productivité spécifique et le taux de croissance. Par contre, une telle relation n'est observée ni pour l'acide propionique ni pour l'acide acétique. Leur production n'est donc pas couplée à la croissance. Ce fait est à rapprocher de l'assimilation précoce du glycérol, précurseur de ces deux acides.

Dans la figure 3, les performances de la fermentation acidogène sont étudiées pour divers taux de dilution appliqués au réacteur. La productivité en acides, atteint 1 g /l de fermenteur /h pour un taux de dilution de 1 j^{-1} (temps de résidence de 1 j). Au delà de cette vitesse, ($D = 2\ j^{-1}$) les substrats de la vinasse sont incomplètement utilisés.

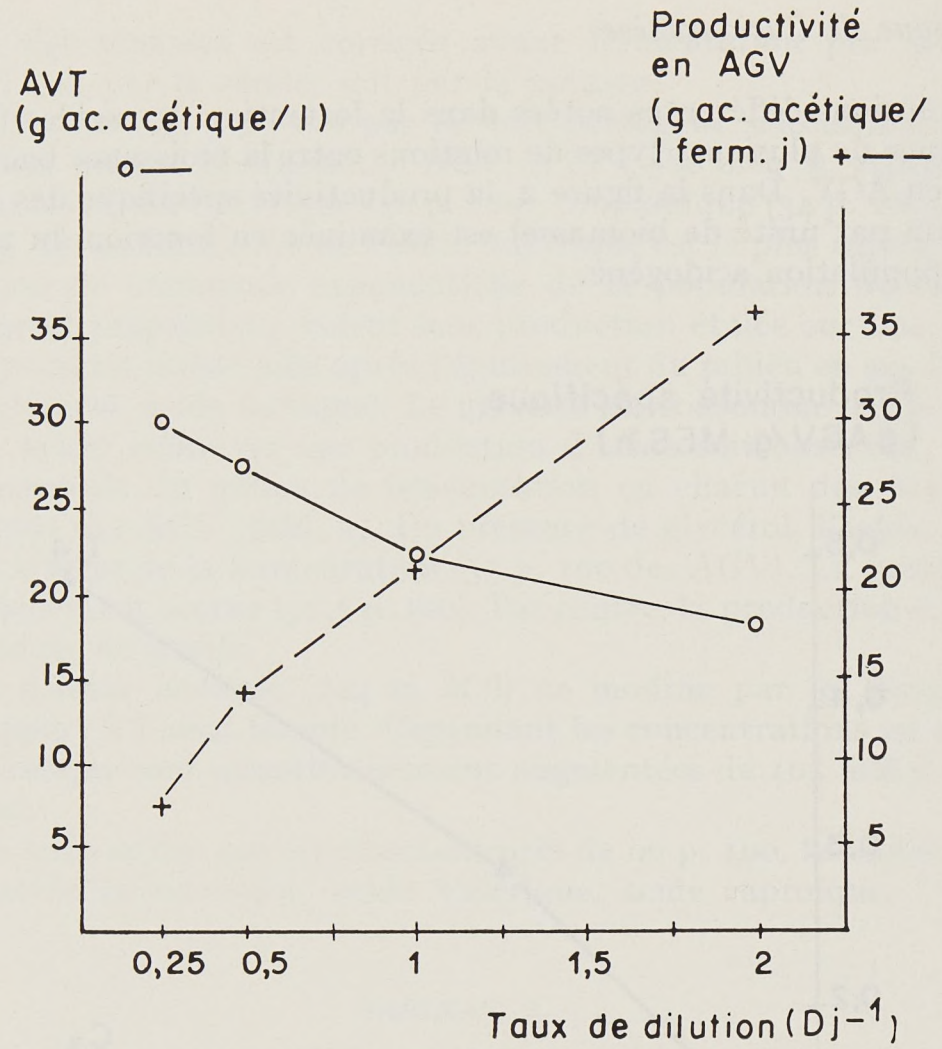

FIG. 3. — *Influence du taux de dilution du fermenteur acidogène sur la concentration et la productivité en AGV*

3. — *Paramètres de la fermentation acidogène*

L'acidification du milieu est observée au cours de cette phase. Le pH diminue jusqu'à pH 6. La formation d'AGV à partir du glycérol est responsable de ce phénomène. A partir d'acide lactique, salifié dans la vinasse, la production d'AGV ne devrait pas influencer ce paramètre. L'agent de neutralisation utilisé pour corriger le pH de la vinasse avant fermentation peut être aussi bien la soude que la potasse. Cependant, la soude s'avère préférable; elle présente l'avantage d'équilibrer la composition minérale de la vinasse dont le potassium est l'élément dominant (tabl. 1). En fermentation continue, la neutralisation de la vinasse à pH 7 permet de maintenir le milieu de fermentation à pH optimal de 6,2.

Les gaz formés en phase acidogène sont le gaz carbonique et l'hydrogène. En fermentation discontinue, le dégagement d'hydrogène est superposé à la phase de croissance exponentielle et peut représenter momentanément jusqu'à 50 p. 100 des gaz produits. Cette production ne dure que quelques heures. En fermentation continue, le gaz carbonique constitue plus de 90 p. 100 des gaz formés. Le volume total est de 6-8 l/l de vinasse.

4. — *Bilan du carbone*

L'étude de la répartition du carbone après fermentation acidogène permet de dresser le bilan de la conversion microbienne (fig. 4).

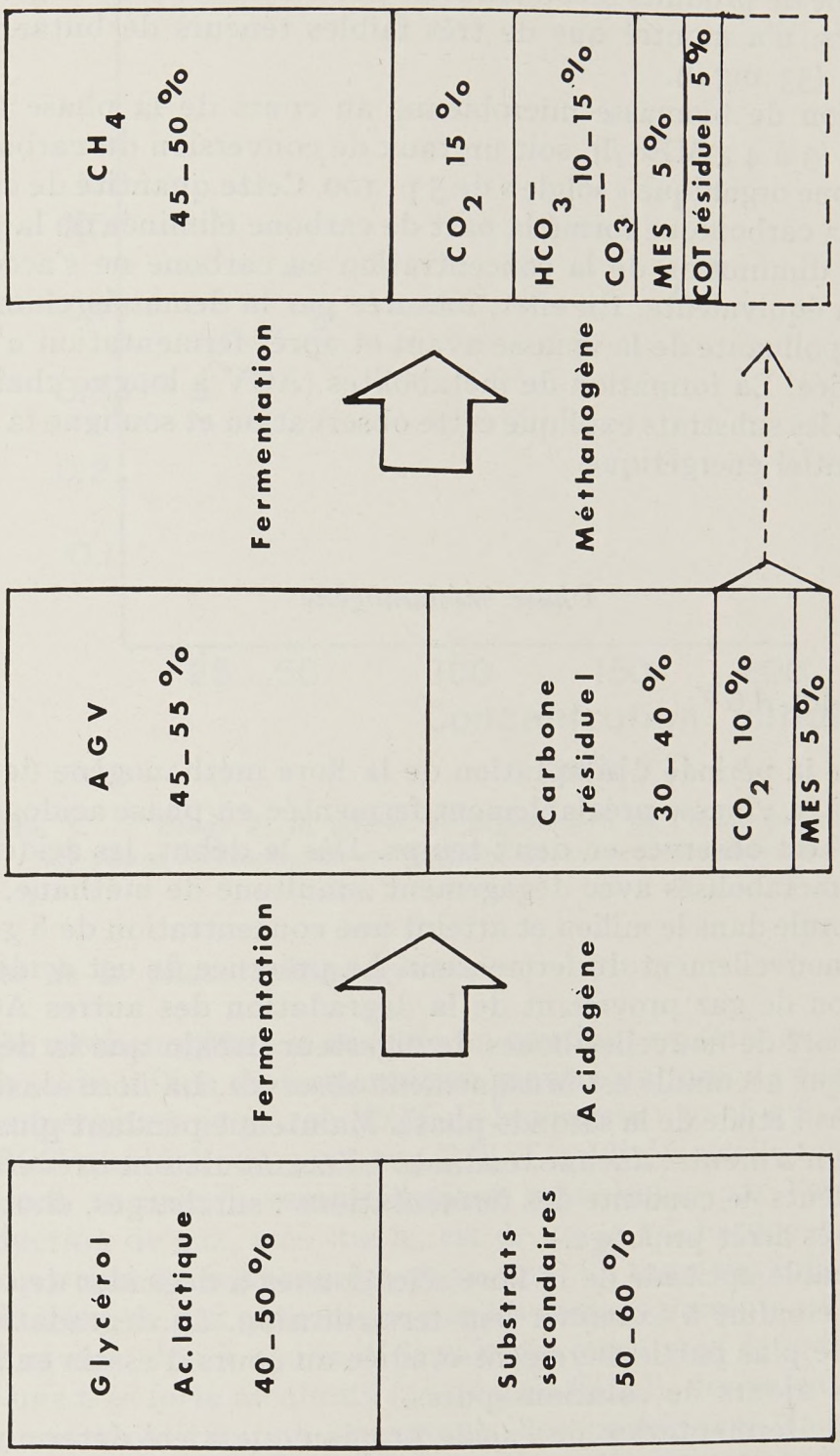

FIG. 4. — *Bilan du carbone de la fermentation méthanique en deux phases de vinasses de vin*

Les AGV formés représentent près de 50 p. 100 du carbone initial contenu dans la vinasse et correspondent essentiellement à la fraction glycérol-acide lactique. L'autre fraction carbonée régresse de 55 à 35 p. 100 par incorporation des composés mineurs dans les AGV et dans la biomasse formée.

La recherche de produits secondaires de la fermentation acidogène, comme les alcools ou esters, n'a montré que de très faibles teneurs de butanol (240 mg /l) et de propanol (53 mg /l).

La formation de biomasse microbienne au cours de la phase acidogène est peu importante (3 à 4 g MES /l), soit un taux de conversion du carbone organique dissous en carbone organique « solide » de 5 p. 100. Cette quantité de carbone représente avec le gaz carbonique formé la part de carbone éliminée de la phase soluble (15 p. 100). La diminution de la concentration en carbone ne s'accompagne pas d'une épuration équivalente. En effet, mesurée par la demande chimique en oxygène, la charge polluante de la vinasse avant et après fermentation n'est pratiquement pas modifiée. La formation de métabolites (AGV à longue chaîne carbonée) plus réduits que les substrats explique cette observation et souligne la quasi conservation du potentiel énergétique.

Phase méthanogène

1. *Dégradation des AGV*

Au cours de la période d'adaptation de la flore méthanogène de digesteur de boues urbaines à la vinasse préalablement fermentée en phase acidogène, la dégradation des AGV est observée en deux temps. Dès le début, les acides acétique et butyrique sont métabolisés avec dégagement simultané de méthane. L'acide propionique s'accumule dans le milieu et atteint une concentration de 8 g /l au bout de la période de renouvellement du fermenteur. La présence de cet acide ne perturbe pas la production de gaz provenant de la dégradation des autres AGV. Ce n'est qu'après un apport de nouvelles boues de digesteur urbain que la dégradation de l'acide propionique accumulé est brusquement observée. La flore ainsi sélectionnée a été utilisée dans l'étude de la seconde phase. Maintenue pendant plus de trois ans, cette population n'a montré aucune tendance à l'accumulation irréversible d'acides malgré des incidents de conduite des fermentations : surcharges, chutes de la température, ou après arrêt prolongé.

La remarquable aptitude de la flore sélectionnée à dégrader de fortes teneurs en AGV nous a conduit à examiner leur fermentation. La dégradation de l'acide propionique a été plus particulièrement étudiée au cours d'essais en fermentation discontinue, par ajouts de solutions pures.

La vitesse de fermentation de l'acide propionique a été déterminée pour différentes concentrations initiales (fig. 5). De fortes teneurs en cet acide (168 mM /l) ne modifient pas sa vitesse de dégradation. Au cours de ces essais, l'analyse des AGV montre qu'aucun acide intermédiaire ne s'accumule dans le milieu. Le bilan gazeux de la fermentation de l'acide propionique a été également suivi, par mesure du volume total produit et analyse de la composition des gaz. Exprimée par rapport à une mole d'acide propionique, la quantité de méthane formée est 1,73 mole.

L'acide acétique, l'acide butyrique, introduits séparément, sont dégradés à des vitesses respectives de 0,5 mM /l.h et 0,4 mM /l.h, semblables à celle de l'acide propionique (0,4 mM /l.h).

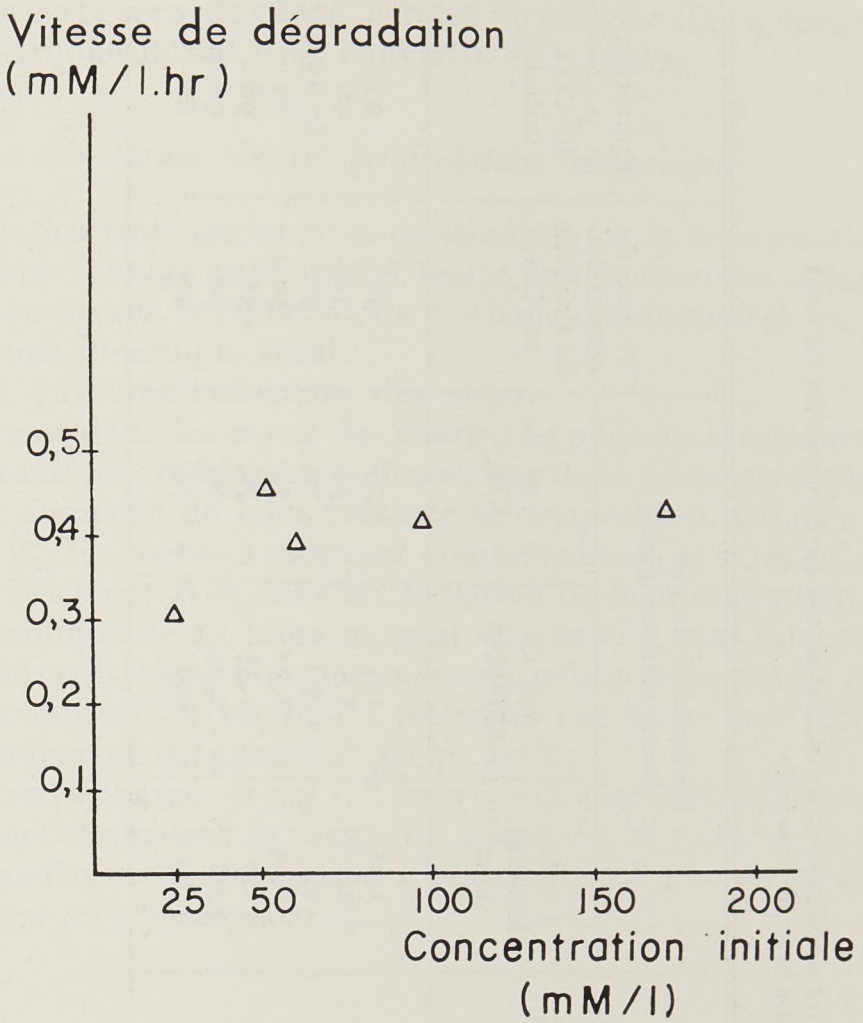

FIG. 5. — *Étude de la vitesse de dégradation de l'acide propionique en phase méthanogène en fonction de sa concentration initiale*

2. *Paramètres de la phase méthanogène*

La phase méthanogène est réalisée en continu, en série avec la phase acidogène. L'analyse du milieu de fermentation montre un taux de dégradation du carbone initial supérieur à 90 p. 100 (COT de la vinasse : 30-36 g /l) atteignant même 98 p. 100, (COT résiduel 600-3 000 mg C /l). L'acidité volatile totale résiduelle est très faible : 2 à 10 méq /l, essentiellement représentée par l'acide propionique.

La production de gaz, très stable, est de 40-45 l /l de vinasse appliquée, avec une forte proportion de méthane (70-75 p. 100). Ce taux élevé de méthane est justifié par la fixation de gaz carbonique par les cations présents en quantités importantes : potassium : 100 à 150 méq /l; calcium : 100 méq /l; sodium : 100 méq /l. Il en résulte une très forte alcalinité (15-20 g Ca CO_3 /l), des valeurs élevées du pH : 8,2-8,5 et un pouvoir tampon important. Secondairement, la nature des AGV (acides à longue chaîne) peut aussi intervenir sur la proportion de méthane par de meilleurs rendements de fermentation.

Les matières en suspension formées dans cette phase sont riches en matières minérales (40-50 p. 100 des matières sèches) dont l'élément dominant est le calcium, précipité sous forme de carbonate. Cette composition confère aux boues une très bonne aptitude à la décantation, par formation de flocs très denses.

La fraction azotée résiduelle dans la phase soluble est sous forme ammoniacale : 800-900 mg N-NH_4 /l. Seulement 40 p. 100 de l'azote initial sont utilisés en fermentation.

TABLEAU 3

Performances de la fermentation méthanique en deux phases appliquée aux vinasses de vin (préconcentrées ou non) en fonction des paramètres de conduite

Essai	Temps de séjour hydraulique (j)		Charge volumique appliquée (g C/l.j)	Temps de rétention des solides (j) en 2e étage	AVT résiduelle (g CH_3COOH/l)	Production de gaz en phase méthanogène			P. 100 de conversion du Carbone en CH_4	Épuration en p. 100 du COT initial
	1re phase	2e phase				1/l de ferment. jour	1/g C appliqué	p. 100 de CH_4		
E_1	2	40	0,65	40	0,38	1,05	1,4	75	56	95
E_2	2	20	1,3	20	0,30	2,25	1,5	73	52	96
E_3	2	13	2,0	13	1,2	2,1	0,9-1	70	40	80-85
E_4	2	13	0,65	13	1,17	0,94	1,24	75	50	89
E_5	2	13	0,65	25-30	0,2	1,0	1,33	72	51	96
E_6	2	13	1,3	25-30	0,18	2,02	1,34	68	50	98
E_7	2	6,6	1,3	6,6	0,8-1,0	1,2-1,5	0,9	73	40	85

E^1, E_2, E_3 : vinasse préconcentrée.
E_4, E_5, E_7 : vinasse préconcentrée, diluée 3 fois.
E_6 : vinasse préconcentrée, diluée 2 fois.

A l'issue de cette seconde phase, le bilan du carbone (fig. 4) montre que près de 50 p. 100 du carbone initial sont convertis en méthane.

3. *Performances cinétiques de la fermentation méthanique*

L'influence des trois paramètres de conduite de la fermentation : temps de séjour hydraulique, charge appliquée et temps de rétention des solides est observée sur la production de gaz (proportion de méthane, productivité), sur le taux d'épuration du carbone organique total.

Le tableau 3 résume l'ensemble des essais.

Afin d'obtenir dans les essais des charges appliquées identiques et des temps de séjours hydrauliques différents, l'effluent issu de la phase acidogène est dilué en conséquence. La vinasse de base présente la composition indiquée au tableau 1.

Les essais E_1, E_4, menés à moyenne charge volumique (0,65 g C /l fermenteur. jour soit 2 kg DCO /m3 .j) et sans recirculation de boues montrent que le temps de séjour hydraulique de 13 jours ne peut être atteint sans diminution des performances de fermentation. Pour ces mêmes conditions de charge et de temps de séjour, l'augmentation du temps de rétention des boues par recirculation (E_5) améliore le rendement d'épuration (96 p. 100).

Pour des fortes charges (1,3 g C /l ferm .j soit 4 kg DCO /m3 .j) la recirculation des boues permet d'abaisser le temps de séjour de 20 j (E_2) à 13 j (E_6) tout en obtenant les meilleures performances d'épuration (98 p. 100) et de productivité en gaz (2 m3 gaz /m3 fermenteur.j).

Potentialité énergétique de la fermentation méthanique

La fermentation méthanique libère 0,9 à 1 m3 de méthane /kg Carbone.

Pour une vinasse issue directement de la distillation (non concentrée) dont la charge organique est de 10 g COT /l (DCO : 30 000 mg o_2 /l), la production de méthane se chiffre à 0,9-1 m3 /hl de vinasse, soit l'équivalent énergétique de 33 mégajoules ou 8 thermies.

Dans la figure 6, nous avons intégré la potentialité énergétique de la fermentation des vinasses dans le contexte de la distillation d'alcool de vin et du traitement des effluents par une autre voie d'épuration : l'oxydation biologique. Ce bilan est établi pour la production d'un hectolitre d'alcool pur et les consommations d'énergie de la distillation et de l'épuration aérobie sont issues de données industrielles.

Le traitement d'épuration des vinasses par voie aérobie nécessite pour l'apport d'oxygène une consommation d'énergie de 2,5 Kwh /l de vinasse, (Bories et Maugenet, 1979, Bories *et al.*, 1981), ce qui représente 12 p. 100 de l'énergie dépensée à la distillation.

La fermentation méthanique s'affranchit de ces besoins et produit de plus l'équivalent de 8 Th /hl de vinasse, soit près de 40 p. 100 de la consommation de la distillation.

Le rendement énergétique de la distillation d'alcool

$$\frac{\text{valeur énergétique de l'alcool} - \text{énergie de distillation}}{\text{Valeur énergétique de l'alcool}} = 0{,}66)$$

tel qu'il est présenté à la figure 6, peut être amélioré de 24 p. 100 par récupération de l'énergie produite par fermentation des vinasses.

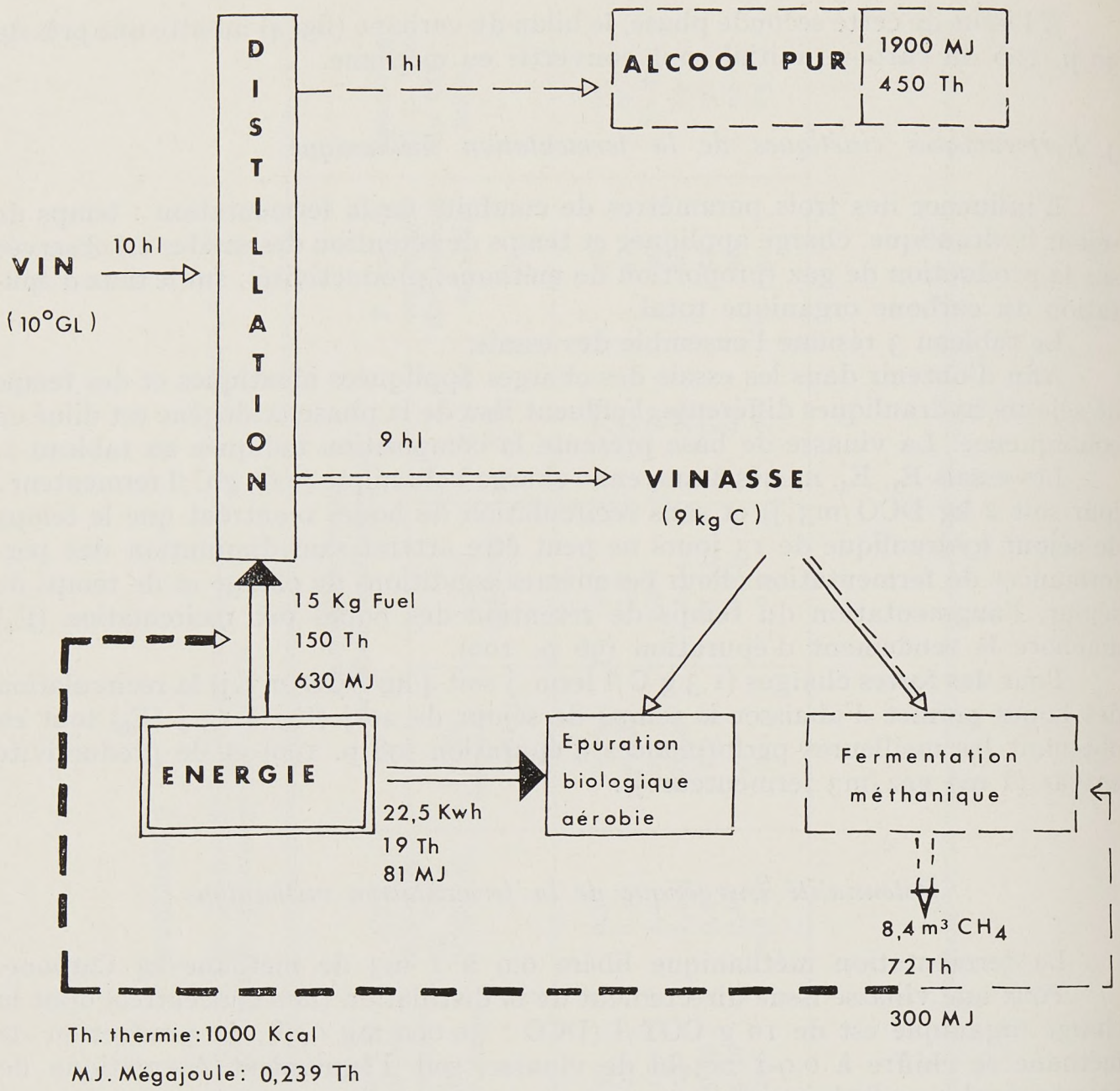

FIG. 6. — *Bilan énergétique de la distillation d'alcool de vin et du traitement des vinasses par fermentation méthanique ou par oxydation biologique*

Ces estimations représentent le cas le plus favorable. Dans ces bilans de fermentation, les besoins énergétiques éventuels pour assurer la conduite des digesteurs (maintien de la température) n'ont pas été pris en compte. Cependant, la récupération d'énergie au niveau des vinasses (100 °C) et des eaux de condenseurs (50 °C) devrait en couvrir une grande part.

Discussion

1. *Comportement des phases acidogène et méthanogène*

A partir de milieux riches en matières organiques solubles, comme l'est l'eau résiduaire de distillerie étudiée (tabl. 1), la phase acidogène s'implante très facilement et assure la conversion complète des principaux substrats en AGV (fig. 1 et 4). La formation d'importantes quantités d'acides (30 à 36 g /l) suggère les risques

d'accumulation encourus en fermentation monoétagée dès la moindre surcharge du milieu ou perturbation de la production de méthane. Sur ce plan, la réalisation d'une phase acidogène séparée est un moyen de contrôler la formation des AGV, d'optimiser leur production, de régulariser leur apport en deuxième phase, d'assurer la stabilité du processus dans son ensemble.

Le profil des AGV formés sur vinasse présente la particularité de réunir les acides butyrique et propionique en forte concentration (70 p. 100 des AGV à eux deux). La nature des substrats mis en jeu : glycérol et acide lactique, mais probablement aussi celle des micro-organismes sélectionnés sont à considérer dans ce résultat. En culture pure et sur milieux simples, la formation de ces acides, par diverses bactéries est bien connue. En ce qui concerne l'acide butyrique, son métabolisme a été décrit par Miller et Jenesel (1979) chez *Butyrivibrio fibrisolvens*. Pour l'acide propionique, deux voies métaboliques différentes ont été identifiées : une dite du succinate chez *Veillonella parvula* (NG et Hamilton, 1973) et *Bacteroïdes fragilis* (Macy *et al.*, 1978); l'autre dite voie acrylique mise en évidence par Leaver *et al.*, (1955) et Wallnofer et Baldwin (1967), respectivement chez *Clostridium propionicum* et *Bacteroïdes ruminicola*.

Un autre point caractéristique du comportement de la phase acidogène réside dans les relations entre la croissance de la flore microbienne et la production des AGV (fig. 2) : couplage pour l'acide butyrique seulement. De la Torre Lozano (1979), Goma *et al.*, (1979) observent le couplage pour l'ensemble des AGV avec la croissance d'une population mixte acidogène cultivée sur saccharose. Ces mêmes auteurs ont analysé la cinétique acidogène et proposé des modèles décrivant l'effet inhibiteur des AGV sur la phase acidogène.

L'étude de la phase méthanogène dégage des enseignements précieux vis-à-vis de l'effet des AGV. La production de méthane en fermentation continue est réalisée à partir d'un milieu contenant 500-600 mM d'AGV /l (AVT : 30 à 36 g /l). L'assimilation de l'acide propionique à forte concentration (168 mM /l) constitue un fait remarquable (fig. 5). Les mécanismes de dégradation des AGV supérieurs en fermentation méthanique sont à ce jour méconnus. Non utilisés par les bactéries méthanogènes isolées jusqu'ici, leur passage par l'acétate est le schéma général proposé. Le butyrate subirait une béta oxydation. Quant au propionate, Scharer et Moo-Young, 1979 suggèrent des mécanismes inverses de la voie de synthèse par le succinate, aboutissant à l'acétate.

2. *Effet et signification des AGV*

L'effet inhibiteur des AGV supérieurs, notamment de l'acide propionique est souvent évoqué, car, dans les systèmes traditionnels (monoétage), l'accumulation d'AGV accompagne les accidents de fermentation. McCarty et Mc Kinney (1961) avancent l'effet de la nature de la fraction minérale salifiant les AGV; Andrews (1969) attribue l'inhibition à la fraction non ionisée des acides. L'acide propionique intervient négativement sur la production de méthane chez *Methanobacterium formicicum* (Hobson et Shaw, 1976).

Dans notre étude, l'effet d'inhibition ne peut pas être retenu. L'accumulation des AGV en fermentation monoétagée serait non la cause d'une inhibition mais la conséquence de l'arrêt de la production de méthane dû à un autre facteur. La mise en évidence d'associations microbiennes conforte cette opinion. Chen et Wolin (1977) montrent que la co-culture de *Selenomonas ruminantum* et *Methanobacterium ruminantum* fermente le lactate en méthane avec formation d'acide acétique

seul. Par contre, en l'absence de la bactérie méthanogène, *Selenomonas r.* produit de l'acide propionique. D'autres exemples d'intéractions microbiennes sont décrits par McInerney *et al.* (1979); Winter et Wolfe (1979). Mc Inerney et Bryant (1979) démontrent la nécessité de telles associations par les niveaux d'énergie de la dégradation des AGV supérieurs et de certains autres intermédiaires.

On peut penser que la non dégradation des AGV au cours d'accidents est due à l'absence d'associations adaptées à ces produits nouvellement formés, et non à leur effet toxique.

3. *Conséquences de la séparation en deux phases*

Ce schéma du métabolisme intermédiaire des AGV peut être discuté également dans le cas de la fermentation en deux phases. La séparation des phases tend à présenter des analogies avec les situations décrivant les accumulations d'AGV dans les accidents de fermentation en monoétage.

L'obtention d'une phase acidogène stricte, en éliminant les bactéries méthanogènes, donc les associations acétogènes-méthanogènes, soit par effet cinétique, soit par des conditions de culture, oriente la production des acides vers les produits à plus longue chaîne carbonée (propionate, butyrate,...). Cette modification de la nature des métabolites intermédiaires nécessitera ultérieurement en phase méthanogène la présence de nouvelles associations spécifiques de ces composés. Mais dès lors que de telles populations sont implantées, le système microbien, par sa nature même, ne devrait plus être sensible au problème de l'accumulation en AGV.

Cette hypothèse s'accorde avec les résultats obtenus dans ce travail. En phase acidogène, les acides propionique et butyrique sont produits massivement. La phase méthanogène, après acclimatation, assure leur dégradation avec production de méthane.

Considérée sous cet aspect biochimique et microbiologique, la séparation des phases présente un nouvel intérêt, s'ajoutant aux avantages cinétiques déjà évoqués (Ghosh et Klass, 1978; Massey et Pohland, 1978).

Les orientations métaboliques et microbiologiques, conséquences de la séparation, apporteraient une meilleure stabilité au processus anaérobie.

4. *Intérêts de la fermentation méthanique en épuration*

Les performances de la fermentation méthanique appliquée aux vinasses de distilleries à très forte charge polluante, montrent des perspectives très intéressantes pour le traitement d'épuration. Les fortes charges appliquées (4 kg DCO/m3.j), sur le système en deux phases avec recirculation des boues, se traduisent par des temps de séjours hydrauliques relativement courts (15 j) étant donné les concentrations de l'effluent traité (DCO : 85 000 mg O_2/l). Une épuration très poussée des matières carbonées (98 p. 100) est parallèlement obtenue. Ces paramètres aboutissent à des productivités en gaz élevées (2 m3/m3 ferm. j).

Ces valeurs se situent parmi les meilleures performances citées dans la littérature concernant la fermentation des effluents agro-alimentaires (Stander *et al.*, 1968; Cillie *et al.*, 1969; Roth et Lentz, 1977; Van den Berg et Lentz, 1977; Knol *et al.*, 1978; Lescure et Bourlet, 1979).

L'influence du temps de rétention des boues en phase méthanogène souligne ce facteur comme prépondérant pour l'optimisation cinétique de la fermentation.

Les procédés assurant les temps de rétention les plus longs, comme les filtres anaérobies (YOUNG et MC CARTY, 1968; CAPRI et MARAIS, 1973; CHIAN et DE WALLE, 1977; GENUNG *et al.*, 1978) ou les systèmes tel le digesteur UASB (LETTINGA *et al.*, 1979) devraient permettre la diminution des temps de séjour hydraulique. Une étude actuellement effectuée sur lit bactérien anaérobie, à garnissage plastique, le laisse supposer.

La confrontation entre la filière anaérobie et la voie aérobie pour le traitement des eaux résiduaires met en évidence la supériorité de la fermentation méthanique dans le cas des effluents à forte charge polluante. Des précédents travaux consacrés à l'oxydation biologique des vinasses nous permettent de dresser une étroite comparaison (BORIES et MAUGENET, 1979 *b*; BORIES *et al.*, 1981).

Au delà d'un seuil de DCO de 20-30 000 mg O_2/l, l'épuration biologique aérobie est limitée. Par contre, la fermentation accepte des plus fortes concentrations, et sa compétitivité sera d'autant plus grande que les charges polluantes seront élevées. Les dimensionnements des installations ne seraient pas supérieurs pour l'anaérobiose.

La composition déséquilibrée en nutriments, caractéristique des eaux résiduaires de distilleries (BORIES, 1978) mais aussi de bon nombre d'effluents agro-alimentaires, n'est pas un handicap pour la fermentation méthanique.

La fermentation méthanique des vinasses caractérisées par un rapport DBO/N = 100/3, n'utilise que 40 p. 100 de l'azote initial par suite d'une production réduite de biomasse. En épuration aérobie, de tels déséquilibres peuvent engendrer des accidents de décantation des boues, et nécessitent des technologies adaptées.

Par contre, la fermentation méthanique présente quelques inconvénients sur le plan de la qualité de l'épuration et ceux-ci sont trop souvent oubliés. Les teneurs résiduelles en azote, parfois élevées, ainsi que les produits malodorants, appelleraient à un traitement tertiaire pour atteindre la qualité la meilleure.

En ce qui concerne l'aspect énergétique, le bilan très favorable de la fermentation méthanique en fait un système d'épuration autonome, voire producteur d'énergie, l'excèdent pouvant améliorer de façon non négligeable le bilan de la production d'alcool (fig. 6).

Conclusion

La dissociation de la fermentation méthanique en deux phases distinctes (production d'AGV, production de méthane) s'avère, à plusieurs titres, particulièrement intéressante dans le cas de milieux à forte charge organique soluble comme les eaux résiduaires de distilleries étudiées ici.

Grâce à la très grande activité de la phase acidogène, la conversion complète des principaux substrats est effectuée très rapidement, d'où l'intérêt premier de sa séparation pour contrôler la formation des AGV et éviter leur accumulation ultérieure. Chacune des phases est réalisée dans les meilleures conditions, ce qui conduit à l'optimisation du procédé.

L'analyse du métabolisme intermédiaire des AGV est accessible par cette technique. Le comportement de la phase méthanogène vis-à-vis des AGV révèle sous un nouvel angle leur signification et effet. Le phénomène ambigu de la toxicité attribuée aux AGV semble devoir être écarté à la vue des résultats concernant la

dégradation de l'acide propionique notamment. L'interprétation des mécanismes biochimiques et microbiologiques récemment acquis peut être appliquée au processus de la séparation des phases et suggère des conséquences non soulignées jusqu'ici. L'orientation des métabolismes et des populations microbiennes que provoquerait la séparation des phases constitue un moyen d'intervention sur le comportement de la fermentation pouvant aboutir à un contrôle et une stabilité meilleurs.

La faisabilité de la fermentation méthanique dans le traitement des effluents de distilleries est confirmée par les performances obtenues. La cinétique et le rendement d'épuration du processus anaérobie sont comparables et même supérieurs à ceux que peuvent présenter la voie par oxydation biologique. Pour les fortes charges, seule la filière anaérobie est envisageable. Les temps de séjours relativement courts font envisager des volumes de fermentation acceptables. L'aspect le plus intéressant concerne le potentiel énergétique de la fermentation des effluents, qui non seulement peut assurer l'autonomie énergétique de l'épuration, mais aussi apporter une valorisation énergétique des déchets. La fermentation méthanique représente une solution séduisante au problème du traitement des rejets liés à la production d'éthanol, secteur promu à d'importants développements.

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Summary

Methane fermentation with separation of acidogenic and methanogenic stages applied to treatment of high strength wastewaters (distilleries)

The methane fermentation with phase separation (acidogenesis and methane production) is studied on high strength distiller wastewater (wine stillage, COD : 85 000 mg O_2/l). The study of two phases reveals intermediate metabolism of volatile fatty acids (VFA). This process appears to be a means of controlling and carrying out fermentation.

In acidogenic phase, a high VFA production is observed (TVA : 580 méq/l, 35 g/l as acetic acid). Butyric (35-40 p. 100), propionic (30-35 p. 100), acetic (15-25 p. 100) acids are major products obtained from wastewater substrates: such as glycerol and lactic acid. The acidogenic kinetic and the results of conversion into VFA are investigated.

In methanogenic phase, microbial populations wich have been adapted to this VFA profile carry out complete degradation of acids as well as secondary substances.

No inhibition on the methane production by propionic acid is observed, even at high concentration (168 mM/l).

The waste treatment performance evaluation is very high (98 p. 100). The kinetic parameters : hydraulic residence time, loading rate, solids retention time and methane productivity are examined on mixed fermenters.

Energy evaluation to methane fermentation of wine stillage is compared to treatment process by biological oxidation. Besides, energy potential of waste fermentation may act as 40 p. 100 of energy needs for alcohol production.

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Colloque international sur la stimulation électrique et le désossage à chaud des carcasses

CLERMONT-FERRAND
14 au 16 octobre 1980

Le colloque sur la stimulation électrique et le désossage à chaud des carcasses s'est déroulé du 14 au 16 octobre 1980 à Clermont-Ferrand. Il était organisé par l'Institut National de la Recherche Agronomique et l'Association pour le Développement de l'Institut de la Viande, sous le patronage du Secrétariat d'État aux Industries Agro-Alimentaires et sous l'égide de l'Office National Interprofessionnel du Bétail et des Viandes.

Ce colloque a réuni près de 200 personnes de différents pays et des différents milieux concernés par ces nouveaux aspects de la technologie de la viande, milieux scientifiques, techniques, administratifs et professionnels. Les communications présentées à ce colloque et publiées, dans la langue originale de leur présentation, constituent la mise à jour la plus récente de nos connaissances dans ce domaine. Elle sera utile à tous ceux qui s'intéressent à l'industrie de la viande.

B. L. Dumont.

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Stimulation électrique et désossage précoce des carcasses, problèmes d'actualité pour la filière viande

Allocution d'ouverture du colloque

B. L. DUMONT

I.N.R.A., Laboratoire de Recherches sur la Viande,
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Le colloque auquel nous allons participer pendant ces trois prochains jours est consacré à l'étude des problèmes de différentes natures que posent présentement les possibilités d'application de l'excitation électrique des carcasses et de leur désossage ultraprécoce.

Ces deux sujets constituent des thèmes importants de préoccupation pour chacun dans la filière viande, quels que soient la place qu'on y occupe et le rôle qu'on y joue. Ce sont des thèmes d'actualité, évoqués fréquemment dans la presse scientifique et technique et sur lesquels les informations disponibles sont dispersées, disparates et fragmentaires. Les conséquences de ces techniques sont certainement importantes mais encore mal définies et parfois discutées.

Étrange destinée que celle de la technique de la stimulation électrique des carcasses!

C'est probablement en 1663 qu'on démontra pour la première fois qu'on pouvait provoquer la contraction d'un muscle en stimulant son nerf par une action mécanique. GALVANI plus de 100 ans après le confirma. Benjamin FRANKLIN, l'inventeur du paratonnerre, électrocutait ses dindons pour les tuer et constatait déjà — c'était vers 1750 — que leur viande était extrêmement tendre.

Le temps avait largement passé lorsqu'en 1951 on vit apparaître un brevet américain de HARSHAM et DEATHERAGE indiquant les effets bénéfiques sur la tendreté de la viande d'une stimulation des carcasses de bovin. Peu de suites immédiates furent données semble-t-il à ce travail, et ce n'est qu'à partir de 1973 que, selon l'expression de BENDALL, l'excitation électrique fut « ressuscitée » par les chercheurs néo-zélandais qui la mirent alors en pratique dans leurs usines, d'abord pour les agneaux, puis pour le bœuf.

Il est vrai qu'alors l'intérêt académique que la stimulation électrique avait pu avoir jusque là se changeait en un intérêt pratique capital puisque son apparition pouvait pallier les difficultés qu'on rencontrait, sur le plan de la tendreté des viandes, dans l'application d'une réfrigération précoce.

Le durcissement de la viande refroidie trop rapidement imposait — et impose

toujours — des remèdes préventifs qui soient à la mesure de l'importance que possède la tendreté comme facteur de la qualité des viandes.

La précocité du désossage et sa pratique alors même que la température corporelle est encore très élevée et qu'on travaille alors sur des chairs encore toutes chaudes n'a pas laissé de trace marquante dans l'histoire de la technologie carnée. Il est probable et même certain que les premiers hommes ont dû recourir à l'utilisation précoce de la chair des animaux qu'ils tuaient à la chasse puisqu'ils n'avaient pas de moyens de conservation. L'homme ne s'est réellement affranchi de cette nécessité d'un désossage précoce en vue de l'utilisation de la viande fraîche qu'avec le développement de la réfrigération. Alors même que, dans tous les pays industrialisés, les moyens techniques existent maintenant pour permettre, grâce au froid, une stabilisation des viandes et leur utilisation différée, le bien-fondé d'un report, dans le temps, des opérations de désossage et de préparation des viandes est néanmoins remis en cause. Pour des raisons d'économie, d'efficacité et de meilleure valorisation des produits animaux.

L'excitation électrique des carcasses est-elle réellement nécessaire? Qu'en est-il du désossage à chaud?

Nous sommes arrivés à un point dans l'analyse de ces deux problèmes où il est apparu absolument indispensable de provoquer la réunion de tous ceux qui sont intéressés ou impliqués dans la mise au point, le développement et l'application de ces nouvelles techniques.

Pour leur part, les scientifiques ont d'abord à faire devant nous le point des connaissances acquises et des faits parfaitement établis qui définissent les principes et les bases des techniques à employer et des opérations à mettre en œuvre. Mais ils ont surtout à préciser les limites actuelles de nos connaissances et à bien définir les zones d'incertitude où la recherche est nécessaire pour progresser. Il faut aller dans ce domaine jusqu'au niveau des mécanismes ultimes et ne pas hésiter à tout évoquer malgré le caractère difficile que peut avoir, parfois pour un large auditoire de non-spécialistes, l'exposé de thèses scientifiques.

L'emploi des techniques de stimulation des carcasses et de désossage à chaud suppose aussi une analyse détaillée préalable des conditions pratiques d'utilisation et de leurs facteurs limitants, au plan de la réglementation, de la sécurité, de l'organisation générale de l'entreprise. Également, sera-t-il nécessaire d'apprécier les conséquences de tous ordres de l'utilisation de ces techniques qui laissent prévoir, par leurs incidences possibles sur l'économie et sur la texture de la filière, des changements d'une grande importance. C'est le moins qu'on en puisse dire.

Déjà en France des essais ont été réalisés et des équipements installés. D'autres pays ont aussi, dans ces domaines, une expérience personnelle particulière adaptée à leurs problèmes particuliers, mais dont la connaissance est pour nous, par la généralisation que nous pouvons en faire, du plus grand intérêt. Le témoignage des experts étrangers est, de ce point de vue, essentiel.

C'est pour aborder ces problèmes de la façon la plus complète possible, au plan scientifique, technique, pratique, économique, hygiénique et social et en tenant compte de ce qui se fait dans le monde entier que ce colloque a été organisé à l'initiative des chercheurs en viande de l'I.N.R.A.

Dès qu'ils ont fait part de leur projet, ces chercheurs de l'I.N.R.A. ont reçu un accueil favorable, et je dirai même très favorable, de tous ceux dont la collaboration et la coopération étaient nécessaires à la réalisation d'une pareille entreprise. Un directoire scientifique s'est constitué, comprenant autour de moi MM. Boccard, Frouin, Rosset et Valin, et a bâti dans le détail le programme du colloque.

L'A.D.I.V. a pris en charge, à l'initiative de son président, l'organisation technique et matérielle de la réunion.

Les organisateurs ont eu comme souci principal de présenter à cette réunion sur chaque sujet l'expérience, l'opinion, le point de vue de spécialistes de classe internationale de manière à bénéficier de toute la connaissance disponible à ce moment et d'éclairer le sujet le plus complètement possible. Nous pourrons ainsi bénéficier du concours de collègues des différents pays d'Europe (Allemagne, Belgique, Danemark, Italie, Grande-Bretagne, Norvège, Suède), des États-Unis et de Nouvelle-Zélande.

Les organisateurs ont confié à un animateur particulier le soin de présider les réunions de chacune des trois journées, d'ordonner la discussion et d'intervenir personnellement pour orienter et relancer les débats.

Nous devons être reconnaissants aux experts qui ont bien voulu accepter cette charge d'animateur de séance, à savoir MM. ETHERINGTON, ROSSET, FROUIN et PEROL.

Le Dr ETHERINGTON, chef de la section Biochimie au Meat Research Institute de Langford (Grande-Bretagne), dirigera les travaux de la première session où seront évoqués les problèmes suivants :

— Rappels des mécanismes de la rigor mortis et de la maturation des viandes, par C. VALIN.
— Les conditions d'une stimulation électrique post-mortem efficace, par C. L. DAVEY et B. B. CHRYSTALL (*).
— Le catabolisme de l'A.T.P. pendant la stimulation électrique : influence du type métabolique et contractile, par G. MONIN.
— Les effets de la stimulation électrique sur les qualités de la viande, par T. R. DUTSON *et al.* (*).
— La stimulation électrique à bas voltage des carcasses de bœuf en Suède, par H. RUDERUS et S. FABIANSSON (*).

Le Dr ROSSET, directeur du C.N.E.R.P.A.C., animera la session du 15 octobre qui comprendra des démonstrations pratiques de stimulation à l'abattoir de Clermont-Ferrand et la série des exposés suivants :

— Caractéristiques et utilisation des viandes avant la rigor, par K. O. HONIKEL, Chr. FISCHER et R. HAMM (*).
— Microbiologie de la viande chaude, par J. FOURNAUD.
— Les impératifs de la stimulation, par P. SALE.
— Quelques résultats français de stimulation électrique et de désossage à chaud, par J. SORNAY.
— Stimulation électrique des carcasses de taurillons : utilisation de différents voltages et relation entre l'attendrissage et la fragmentation des protéines, par D. DEMEYER et F. VANDENDRIESSCHE (*).

La séance de jeudi 16 octobre au matin sera présidée par M. FROUIN, directeur des Services de Recherches Appliquées de la Société OLIDA, et portera sur les aspects suivants :

— Scénario du futur pour l'organisation de l'industrie de la viande avec la stimulation électrique et le désossage précoce des carcasses, par B. L. DUMONT.

Note: Les conférences suivies de (*) sont publiées en anglais, langue de leur presentation originale au colloque.

— L'expérience britannique de la stimulation électrique et de la transformation à chaud, par A. CUTHBERTSON (*).

— L'application industrielle de la stimulation électrique à bas voltage en Suède, par H. RUDERUS et A. BERGQUIST (*).

— La viande de bœuf désossée à chaud, par A. PETERSEN (*).

— Morceaux typiques italiens obtenus par désossage précoce, par G. BARALDI (*).

— État actuel de la stimulation électrique en Nouvelle-Zélande, par B. B. CHRYSTALL (*).

— Coopération entre l'industrie et la recherche en vue du développement d'une méthode de production de morceaux de choix de bovins désossés à chaud, par L. BUCHTER (*).

— Appareillage pour la stimulation électrique des morceaux de viande désossés à chaud, par O. S. BRAATHEN.

La séance de jeudi 16 octobre après-midi sera animée par M. PEROL, directeur de l'O.N.I.B.E.V., et sera consacrée aux problèmes suivants :

— Aspect réglementaire de la stimulation électrique et du désossage à chaud des viandes, par G. QUINET.

— Conséquence de la stimulation électrique et du désossage à chaud sur l'agencement de la filière viande, par A. CERLES.

— La sécurité du personnel, par J.-M. VACHERET.

— Les conséquences socio-économiques de la stimulation électrique et du désossage à chaud, par J.-E. DE MIEULE.

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Le colloque qui s'ouvre aujourd'hui est une œuvre collective. Que tous ceux qui ont contribué à sa réalisation en soient remerciés, et en premier lieu tous les rapporteurs et les animateurs. Le dévouement des membres de l'équipe de l'A.D.I.V. est pour beaucoup dans la qualité de son élaboration et de sa mise sur pied. Important aussi a été le support fourni par la Commission des Communautés Européennes qui a permis ainsi la participation à nos débats de collègues de pays voisins. Déterminante et indispensable, enfin, a été pour nous la participation matérielle qui a concrétisé les encouragements et l'égide du Secrétariat d'État aux I.I.A. et l'aide matérielle que nous a apportée le patronage de l'O.N.I.B.E.V.

A chacun merci.

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Brefs rappels des mécanismes de la *rigor mortis* et de la maturation des viandes

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Introduction

La transformation du muscle en viande repose très largement sur des mécanismes biochimiques qui, après la mort des animaux, modifient plus ou moins profondément la composition et la structure du muscle. Les réactions mises en jeu, essentiellement des réactions hydrolytiques, dissipent les réserves énergétiques du muscle (ATP, PC et glycogène) et affectent l'état d'organisation et la structure même des protéines et des différentes fractions lipidiques musculaires. De la cinétique et de l'intensité de ces réactions dépendent très largement la définition des qualités des viandes. Différents facteurs peuvent influer sur le cours de ces transformations, des facteurs intrinsèques au muscle traduisant les influences de l'espèce, de l'âge, du sexe, du type de muscle, des facteurs extrinsèques, à savoir les technologies mises en œuvre et en particulier le régime thermique imposé aux carcasses, ce qui semble assez évident puisque les mécanismes enzymatiques sont particulièrement dépendants de la température.

Nous rappellerons donc brièvement les grands traits des mécanismes connus de la rigor mortis et de la maturation afin de pouvoir mieux appréhender par la suite les modifications apportées à ces transformations par les techniques de stimulation électrique et de désossage à chaud et leurs conséquences au niveau de la définition des caractéristiques organoleptiques des viandes.

I. — La *rigor mortis*

Il s'agit de la phase de l'évolution post mortem du muscle qui correspond, sur un plan physique, à la perte des propriétés d'élasticité du tissu musculaire. Cette transformation correspond sur un plan biochimique à l'hydrolyse du complexe Mg ATP^{-2}, composé qui confère à la structure contractile au repos ses propriétés d'élasticité. Parallèlement à la disparition post mortem de l'ATP on observe, en général, une baisse du pH musculaire.

Un certain nombre de facteurs influent sur la vitesse d'épuisement post mortem du muscle en ATP. Parmi eux, deux paraissent particulièrement importants :

a) le niveau des réserves énergétiques de l'animal lors de l'abattage,

b) la cinétique de réfrigération des carcasses,

et nécessitent un examen plus approfondi si on veut parvenir à une meilleure compréhension des transformations induites par la stimulation électrique et le désossage à chaud des carcasses.

1.1. — *Catabolisme post mortem de l'ATP et acidification du tissu musculaire*

Dans le tissu musculaire au repos et après l'abattage des animaux, l'ATP est constamment et lentement hydrolysé selon une réaction du type :

$$(1) \qquad Mg\ ATP^{-2} + H_2O \xrightarrow[\text{hydrolase}]{} Mg\ ADP^{-1} + Pi^{-2} + H^+$$

Cette réaction libère un proton, donc acidifie le milieu. Dans les premiers instants *post mortem* on peut en première approximation considérer que l'essentiel de cette réaction est assurée par l'activité ATPasique de la myosine.

La réaction est lente, ce qui signifie une libération lente des sites actifs de la myosine des produits de cette réaction, à savoir $Mg\ ADP^{-1}$ et Pi^{-2}.

Dans les instants qui suivent la mort, on constate que le taux d'ATP dans le muscle est constant. En effet, l'ADP formé est rephosphorylé, soit par la phosphocréatine présente selon une réaction du type

$$(2) \qquad Mg\ ADP^{-1} + PC^{-2} + H^+ \longrightarrow Mg\ ATP^{-2} + \text{Créatine}$$

qui consomme un proton et neutralise les effets de la réaction (1), soit par la voie glycolytique activée par la libération de Pi^{-2} qui se produit lors de la réaction (1). Cette voie permet, à partir de la consommation d'une unité glucose du glycogène, de rephosphoryler 3 ADP selon un mécanisme global du type :

$$(3) \qquad 3\ Mg\ ADP^{-1} + 3\ Pi^{-2} + 1\ \text{glucose} + H^+ \longrightarrow 3\ Mg\ ATP^{-2} + 2\ \text{lactate}^{-1}$$

Cette réaction consomme un proton, mais comme initialement les 3 ADP rephosphorylés proviennent d'une réaction qui a libéré 3 protons, le bilan net correspond à la production de 2 protons, d'où l'acidification du tissu musculaire qu'on observe en général lors de l'installation de la rigidité cadavérique.

Lorsque la phosphocréatine est consommée, on constate une diminution rapide du taux d'ATP dans le muscle, la rephosphorylation par la voie glycolytique seule ne peut contrebalancer la consommation par les activités ATPasiques.

Donc il apparaît tout d'abord que la glycolyse qui produit du lactate ne va de pair avec une acidification du milieu musculaire (1 H^+/1 lactate) que si l'ADP, substrat de la glycolyse, provient de l'hydrolyse de l'ATP, sinon le fonctionnement de la voie glycolytique s'accompagnerait d'une alcalinisation du milieu (—1 H^+/2 lactates). En second lieu, on constate que l'amplitude de la chute de pH dépend étroitement des capacités du potentiel de réserve (PC et

glycogène) à entretenir post mortem une hydrolyse d'ATP et que la vitesse de chute du pH dépend directement de la vitesse d'hydrolyse post mortem des liaisons phosphates riches en énergie de l'ATP qui peut, d'après les équations précédentes, s'écrire :

$$d\,(\sim P)/dt = -\,d(PC)/dt + 1{,}5\; d\,(\text{lactate})/dt - 2\; d(ATP)/dt$$

ce qui revient à :

$$V_{(\sim P)} = V_{PC} + 1{,}5\; B.V_{pH} + 2\; V_{ATP}$$

où V_{PC} et V_{ATP} sont les vitesses de disparition de l'ATP dans le muscle *post mortem*, B le pouvoir tampon du muscle, V_{pH} la vitesse de chute du pH.

Maîtriser la vitesse d'hydrolyse *post mortem* de l'ATP permet donc de contrôler la vitesse de chute du pH de la viande. Le déclenchement de contractions musculaires par le biais de la stimulation électrique permet d'accélérer la vitesse d'hydrolyse de l'ATP au niveau des sites ATPasiques de l'appareil contractile, ce qui doit s'accompagner d'une accélération de la vitesse de chute du pH.

1.2. — *Influence de la température sur la vitesse d'hydrolyse post mortem de l'ATP*

D'une façon générale, l'abaissement de la température lors de la réfrigération tend à ralentir les phénomènes biochimiques dont les muscles sont le siège. En effet, deux des mécanismes responsables de l'installation de la rigor mortis, la voie glycolytique et l'activité ATPasique de la myosine non dépendante du calcium, présentent une énergie d'activation élevée de l'ordre de 40 kJ /mole. En fait, dans le cas du mécanisme global de la rigor mortis les choses sont moins simples. Comme BENDALL (1973) l'a montré, on observe en fait que le mécanisme qui assure *post mortem* un turn over de l'ATP, dont l'efficacité est caractérisée par la relation $V_{\sim P} = V_{PC} + 2\; V_{ATP} + 1{,}5\; B.V_{pH}$ ne présente un coefficient de température (Q_{10}) constant et égal à 1,7 qu'entre 38 et 25 °C. Pour des températures inférieures, le Q_{10} décroît lentement, s'annule, puis devient de plus en plus négatif, si bien qu'à + 2 °C les vitesses de transformation, dans le muscle sterno-mandibularis de bovin par exemple, peuvent être aussi élevées qu'à + 15 °C. Or, le Q_{10} de l'activité ATPasique de la myosine non dépendante du calcium est constant et égal à 1,7. L'accélération de la vitesse d'hydrolyse de l'ATP dans le tissu musculaire, que l'on enregistre en dessous d'une certaine température lors d'un refroidissement rapide, traduit la mise en œuvre en dessous de + 10 °C environ d'une autre activité ATPasique, l'activité actomyosine ATPase dont le fonctionnement s'accompagne d'une contraction plus ou moins importante du muscle. Il s'agit du phénomène de la contraction au froid découvert par LOCKER et HAGYARD (1963). En fait, pour qu'il y ait contraction au froid, 3 conditions doivent être remplies, à savoir :

1) qu'il reste de l'ATP dans le muscle;
2) que la température soit inférieure à 12 °C;
3) et cela quand le pH est supérieur à 6, valeur en dessous de laquelle l'activité actomyosine ATPase de type rapide est inhibée, l'activité de type lent étant de toute façon inhibée par le froid.

Si on réfrigère très rapidement une carcasse, compte tenu des coefficients de température élevés des réactions d'hydrolyse de l'ATP, on ralentit considérablement la vitesse de chute du pH, si bien qu'il devient possible d'abaisser la température de certains muscles en dessous de + 10 °C alors que le pH est encore supérieur à 6, ce qui crée les conditions du déclenchement des contractions au froid qui altèrent irréversiblement la tendreté de la viande. En pratique, ces conditions sont rassemblées, même dans le cas de la réfrigération des carcasses des gros bovins, dans les systèmes de réfrigération rapide installés dans les abattoirs modernes (air pulsé à 3 m/sec à la température de — 2 °C pendant 2 h, puis équilibrage dans salle à 0 °C, air brassé à 1 m/sec).

Le deuxième point qu'il convient d'évoquer concerne l'activité actomyosine ATPase qui est mise en jeu d'une façon générale lorsqu'il y a contraction musculaire. Cette activité enzymatique présente une énergie d'activation très élevée (100 kJ/mole). Cela signifie en particulier que si on déclenche post mortem des contractions musculaires par le biais de stimulations diverses, par exemple à la suite d'une stimulation électrique, la consommation d'ATP par cette enzyme et par conséquent la chute de pH qui accompagneront ces contractions seront d'autant plus importantes que la température du muscle sera plus élevée, comme l'ont souligné récemment Chrystall et Devine (1980).

Pour conclure ce bref exposé sur les mécanismes d'installation de la rigor mortis, il convient de souligner outre l'influence du facteur température, le rôle majeur joué par les structures contractiles sur la cinétique de ces transformations.

II. — La maturation

Il s'agit d'une phase de l'évolution *post mortem* très complexe qui dure en fait tout le temps qui sépare l'abattage des animaux de la consommation de la viande. Si on restreint la *rigor mortis* au catabolisme *post mortem* des réserves énergétiques du muscle, la maturation par contre couvre l'ensemble des modifications qui vont affecter après la mort des animaux la structure et la nature même des composants du muscle. Compte tenu de l'orientation de ces journées, nous limiterons notre propos aux problèmes de l'attendrissage post mortem des viandes, mais il faut bien avoir présent à l'esprit que c'est l'ensemble des caractéristiques organoleptiques de la viande qui est affecté par le phénomène de maturation (tendreté, couleur, jutosité, saveur).

La *rigor mortis* correspond à la perte des propriétés d'élasticité du tissu musculaire, ce qui va de pair avec un durcissement de la viande. Par la suite, au cours de la maturation, la tendreté s'accroît progressivement. Cet attendris-au cours de la maturation, la tendreté s'accroît progressivement. Cet attendrissage correspond à une évolution des propriétés mécaniques du tissu musculaire qui résulte essentiellement de modifications intervenant au niveau de la structure myofibrillaire, induites très vraisemblablement par les systèmes protéolytiques intramusculaires. Nous examinerons successivement la nature de ces modifications ainsi que le rôle possible des systèmes protéolytiques musculaires, puis nous discuterons de l'effet de quelques facteurs de variation de la vitesse de maturation de la viande.

2.1. — *Modifications de la structure myofibrillaire*

De très nombreuses études ont été consacrées à la description des modifications qui affectent la structure myofibrillaire en cours de maturation. Globalement, on peut en résumer les principaux résultats à :

— une destruction progressive de l'image des stries Z allant de pair avec l'exclusion de l'α-actinine de la structure myofibrillaire;

— un affaiblissement des interactions protéine-protéine accompagné d'une évolution des propriétés de solubilité des protéines myofibrillaires et d'une modification de certaines activités ATPasiques du système;

— une attaque protéolytique caractérisée d'un composant des filaments fins, la troponine T.

Ces modifications s'accompagnent d'une fragilisation progressive de la structure myofibrillaire, contemporaine de l'augmentation de la tendreté. Cette fragilisation peut être appréciée par des tests mécaniques tel le MFI (myofibrillar fragmentation index) qui est corrélé significativement avec la tendreté (MOELLER *et al.*, 1973) ou bien avec l'évolution du travail de cisaillement du muscle cru (GOUTEFONGEA, VALIN, 1978). En dépit du nombre de travaux existant il demeure de nombreuses inconnues, tant au niveau de l'inventaire des modifications structurales de la structure contractile qu'en ce qui concerne l'existence d'une relation quantitative directe entre les modifications observables de la structure myofibrillaire et l'évolution de la tendreté en cours de maturation. Pour l'instant, on ne peut guère utiliser les modifications quantitativement mesurables de ces structures telles par exemple l'hydrolyse de la troponine T ou l'évolution de l'effet de la force ionique sur l'activité actomyosine ATPase Mg Ca dépendante que comme des indicateurs de maturation et non comme des mesures de tendreté, mesures qui n'auraient de toute façon de sens qu'à teneur en collagène constant.

2.2. — *Les systèmes protéolytiques musculaires*

Deux systèmes sont supposés intervenir dans ces transformations, tout d'abord une protéase neutre activée par le calcium, le CAF découvert par BUSCH *et al.* (1972) et purifié récemment par l'équipe de DUCASTAING, et d'autre part les enzymes lysosomales actives à pH acides.

2.21. — *Le CAF*

Cette enzyme a la propriété *in vitro* de détruire la strie Z et la bande M, d'hydrolyser la troponine T et, comme nous l'avons vérifié récemment, de modifier l'activité ATPasique Mg Ca dépendante d'une façon tout à fait identique à l'évolution subie par cette activité en cours de maturation. Est-ce à dire que cette enzyme est responsable de ces transformations en cours de maturation, évidemment non. Ainsi, le CAF qui hydrolyse la troponine T devrait provoquer une augmentation de l'activité ATPasique Mg EGTA dépendante, ce qui n'est pas le cas alors qu'on observe effectivement en cours de maturation une augmentation de cette activité enzymatique.

Il est en effet impossible d'extrapoler directement des résultats obtenus *in vitro* aux mécanismes existant *in situ*. Plusieurs questions restent posées quant à la participation du CAF aux mécanismes de la maturation, d'une part cette enzyme est activée *in vitro* par le Ca^{++} à des concentrations très supérieures à celles existantes *in situ;* en second lieu, on a découvert et purifié récemment un inhibiteur sarcoplasmique du CAF (Cottin *et al.*, 1980) et on peut s'interroger sur le mécanisme de régulation de l'activité de cette enzyme aussi bien *in vivo* que *post mortem*.

2.22. — *Les enzymes lysosomales*

Considérées pendant très longtemps dans les manuels comme responsables de l'évolution *post mortem* de ces structures, ce n'est que très récemment que des preuves expérimentales d'une action *post mortem* de ces enzymes sur la structure contractile ont été apportées.

On sait maintenant que les cathepsines, au moins B et D, peuvent dégrader la myosine, l'actine et la troponine T (Okitani *et al.*, 1977; Schwartz et Bird, 1977) et nous avons pu également induire *in vitro* des modifications des activités ATPasiques similaires à celles observables *in situ* en incubant des myofibrilles avec le système lysosomal (Ouali, Valin, 1980). Il semble de plus que ce sont les thiols protéases lysosomales qui joueraient un rôle majeur dans ces transformations comme d'ailleurs l'avaient suggéré Penny et Ferguson Pryce (1979). Comme pour le CAF, plusieurs questions restent en suspens quant à l'intervention *post mortem* de ces protéases. Pour que ces enzymes soient efficaces *post mortem*, il faut :

— qu'elles soient libérées dans la fibre musculaire;

— que les inhibiteurs sarcoplasmiques de ces enzymes ne bloquent pas leur activité;

— que les protéines myofibrillaires soient au moins partiellement dénaturées.

Autant de problèmes qui font actuellement l'objet d'études approfondies.

2.3. — *Influence de la température sur la vitesse de maturation*

La vitesse de maturation dépend de l'effet de plusieurs facteurs tels le type de muscle, l'espèce et la température. Chez les bovins, entre muscles et entre animaux on peut observer des différences de vitesse de maturation allant de 1 à 3, toutes choses égales par ailleurs. Entre espèces, on peut considérer que les viandes de bovin, mouton, lapin maturent à des vitesses comparables, les viandes de porc plus rapidement. Enfin, on le sait depuis très longtemps, la température des muscles joue un rôle déterminant sur leur vitesse de maturation. Davey et Gilbert (1976) ont montré sur muscle sternomandibularis de bovin qu'entre 0 et 40 °C le Q_{10} de la réaction d'attendrissage est élevé et égal à 2,4, l'enthalpie d'activation étant de l'ordre de 60 kJ. A partir des résultats de Busch *et al.* (1967), nous avons pu calculer des valeurs de Q_{10} du même ordre pour d'autres muscles (psoas, semitendinosus). Peut-on trouver des relations du même ordre entre des modifications intervenant au niveau de la structure myofibrillaire et la température de conservation? Apparemment oui, quand on dispose d'une mesure quan-

titative d'appréciation d'une modification de la structure myofibrillaire. On s'aperçoit alors que ces modifications sont caractérisées par une enthalpie d'activation élevée.

	Δ H	RÉFÉRENCES
Effet de l'osmolarité sur l'évolution de l'activité ATPasique Mg Ca dépendante	49 kJ (muscles)	VALIN, 1978 entre 0 et 15 °C
Solubilité protéines myofibrillaires . .	53 kJ (muscles)	VALIN, 1978 entre 0 et 15 °C
Hydrolyse de la troponine T	72 kJ (muscles)	PENNY, DRANSFIELD, 1979 entre 5 et 35 °C
	37 kJ (homogenat)	PENNY, FERGUSON-PRYCE, 1979 entre 5 et 35 °C

Sans préjuger du ou des mécanismes enzymatiques mis en jeu, il faut retenir cet effet considérable du facteur température sur la vitesse de maturation. Ceci implique que pour un muscle donné un écart relativement faible de température doit conduire à l'observation de vitesse très différentes de maturation, toutes choses égales par ailleurs.

Conclusion

Ces brefs rappels des mécanismes de la rigor mortis et de la maturation avaient surtout pour but de souligner la très grande importance du facteur température et de l'histoire thermique de la carcasse sur le déroulement des phénomènes biochimiques qui conditionnent en définitive les caractéristiques qualitatives des viandes. Nous ignorons beaucoup de choses concernant ces transformations, ce qui nous empêche d'en maîtriser le cours et surtout d'en maîtriser la variabilité. Ce dernier point est de la plus haute importance dans le cadre d'un développement industriel de la filière viande. Cependant, il apparaît que l'on commence à bien connaître les effets de la température sur ces transformations. Si on utilisait déjà couramment ces connaissances, un bon nombre des problèmes qui se posent aux industriels serait partiellement résolu.

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Conditions for an efficient *post-mortem* electrical stimulation

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I. — Introduction

The economic histories of the countries of the Southern Hemisphere owe much to their meat industries and especially to the advent last century of large-scale refrigeration practice. New Zealand, with its isolation, its relatively small size and its high reliance on agriculture, is still critically dependent on a vital meat industry, selling frozen meat to significant markets in the Northern Hemisphere.

Little wonder that it's supportive technology has been concerned with presenting frozen meat as an acceptable alternative to fresh meat. As the world's largest exporter of lamb, New Zealand has a special interest in developing a comprehensive technology, not only in the efficient production of sheep meat, but also in its further processing into high-quality retail products. Of all the qualities of meat, by far the greatest attention has been given in New Zealand to determining factors which affect the tenderness of frozen meat. Present knowledge on the tenderness of meat, particularly from cattle and sheep, appears in two recent reviews (Locker *et al.*, 1975; Davey and Winger, 1980). The New Zealand experience has been gained solely on these species and the significance of electrical stimulation on their processing is described here. Since the emphasis of the work has been to resolve problems of toughness development during both chilling and freezing, using rather special methodology, most attention is given in this paper to the work done in New Zealand. It is with no parochial intent that a lesser mention is made of the excellent investigations undertaken in other laboratories on other muscles and species.

II. — Changes associated with the development of rigor mortis

The merit of electrical stimulation in meat technology is intimately associated with the development of *rigor mortis* in muscle, with the cold shortening phenomena, with thaw rigor and with meat aging. Brief attention is given to these topics in preparation for the discussion on electrical stimulation which follows.

A. — *Rigor Mortis*

After an animal dies its skeletal muscles live on in a pre-rigor state. The muscles can be excited to contract and do work and are reversibly extensible. If starved of oxygen they enter *rigor mortis* some minutes or hours *post mortem* and become non-excitable and rigid. From extensive studies of rigor development (BENDALL, 1960), it is evident that the biochemical changes are likely to be the same for all species, with the complete disappearance of ATP and creatine phosphate and, as the product of glycolysis, the appearance of lactate ions (up to 100 μmol/g muscle). The ultimate pH of muscle is inversely related to the accumulation of the lactate ions, pH being relatively high (approximately 7.0) in excessively exhausted animals and low (pH 5.5) in well-fed and rested animals.

B. — *Shortening*

A pre-rigor muscle usually shortens slightly on excision to reach its equilibrium length if held in the horizontal position. At temperature above about 20 °C the muscle shortens with rigor development. Such rigor or heat shortening, is small at 20 °C (less than 5 p. 100 of the equilibrium length) but rises to as much as 50 p. 100 at 40 °C. Contrary to expectation, muscle also shortens by 50 p. 100 or more prior to and during the development of *rigor mortis*, if held at 2 °C (LOCKER and HAGYARD, 1963). The magnitude of cold shortening increases with falling temperature and is reduced if there is a delay in chilling. In most respects the phenomenon is similar to normal contraction involving de-phosphorylation of ATP especially if cold shortening occurs under load (DAVEY and GILBERT, 1974).

Shortening also accompanies the development of thaw rigor especially in unrestrained muscles (BENDALL, 1960). Active thaw shortening occurs on thawing muscles which have been frozen at any point up to almost full rigor development, although as the ATP concentration in the muscle falls so does the power generated by the shortening. Thaw shortening (greater than 50 p. 100 in strips of excised lamb muscle) still occurs at an ATP concentration of about 0.3 μmol/g muscle (DAVEY and GILBERT, 1976).

To what extent can cold and thaw shortenings occur in a carcass in which most of the skeletal attachments are intact? A few muscles such as the neck muscles are severed during carcass dressing and are likely to shorten. Other muscles, notably the *M. longissimus* remain attached to the skeleton but since most of the constituent fibres insert into flexible subcutaneous epimysium, the muscle is able to shorten. Even muscles that are fixed absolutely at both ends are capable of cold shortening over a part of their length if portions are subjected to a differential chilling rate. This has been demonstrated in restrained ox *M. sternomandibularis* insulated at both ends and held at 2 °C. A zone of marked shortening occurs in the rapidly chilled central region with concomitant stretching at the two insulated ends (MARSH and LEET, 1966).

III. — Toughness

A. — *Measurement*

Because the work to be reported relies on special methodology, particularly in determining cooked-meat toughness, it is briefly described here.

All shear measurements have been obtained with the MIRINZ tenderometer which applies an increasing load across a cooked meat sample at a constant rate (MACFARLANE and MARER, 1966). The blunt wedge of the tenderometer compresses the cooked meat against a base plate, and, at a certain loading determined by the toughness of the meat, a sharp yielding point is indicated on a load-deformation curve. Shearing forces (SF) are measured on a arbitrary scale of 150 units, each unit being approximately equivalent to a stress of 1.7 kPa.

The relationship between SF values and taste panel score is not a linear one. From many correlations of panel scores and SF values, cooked lamb is regarded as being very tender at shearing values below SF 25, tender from SF 25 to SF 40 and undesirably tough if shearing forces rise above SF 40. (McCRAE *et al.*, 1971). The SF threshold for beef is considered to be somewhat lower at SF 35.

B. — *Toughness and Shortening*

The fact that muscle can enter *rigor mortis* at different degrees of shortening is a matter of considerable significance to the meat technologist. Scientific investigation has demonstrated that muscle shortening during the early post-mortem period affects cooked-meat toughness to a remarkable degree (LOCKER, 1960). The force required to shear the cooked *M. sternomandibularis* across the fibre direction rises steeply as muscle shortening increases to 40 p. 100 (MARSH

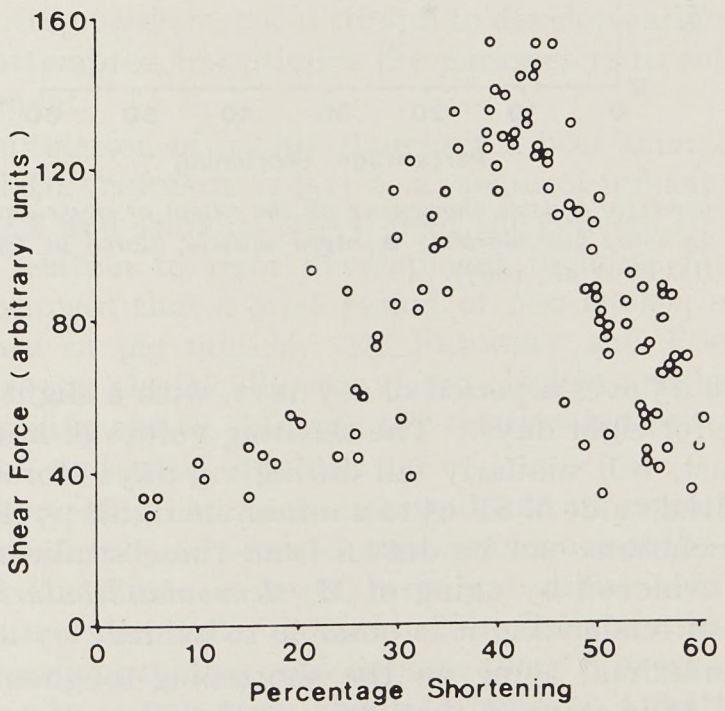

FIG. 1. — *Influence of pre-rigor muscle shortening on shear force of cooked M. sternomandibularis* (after MARSH and LEET, 1966)

and LEET, 1966) (fig. 1). Although of lesser practical significance, with further shortening towards 60 p. 100 the toughness undergoes a sharp decline. Thus a prominent peak of toughness exists in the cooked muscle with its maximum at 40 p. 100 shortening. Cold and thaw shortenings initiated at any stage in the development of rigor produce the same relationship. The peak is encountered in the *M. sternomandibularis* muscles from animals from all maturities (Davey and GILBERT, 1975). These relationships demonstrate that the greatest possible cause of toughening in cooked meat is the shortening produced in pre-rigor muscle at low temperatures. Animal age is merely a secondary cause.

C. — *Meat Aging*

If rigor muscle is aged for a number of days before cooking, it usually becomes appreciably more tender. It appears that the onset of tenderizing begins at about the time muscles enter rigor, although the point needs further investigation. Shear values of unshortened *M. sternomandibularis* for example held at 15 °C fall

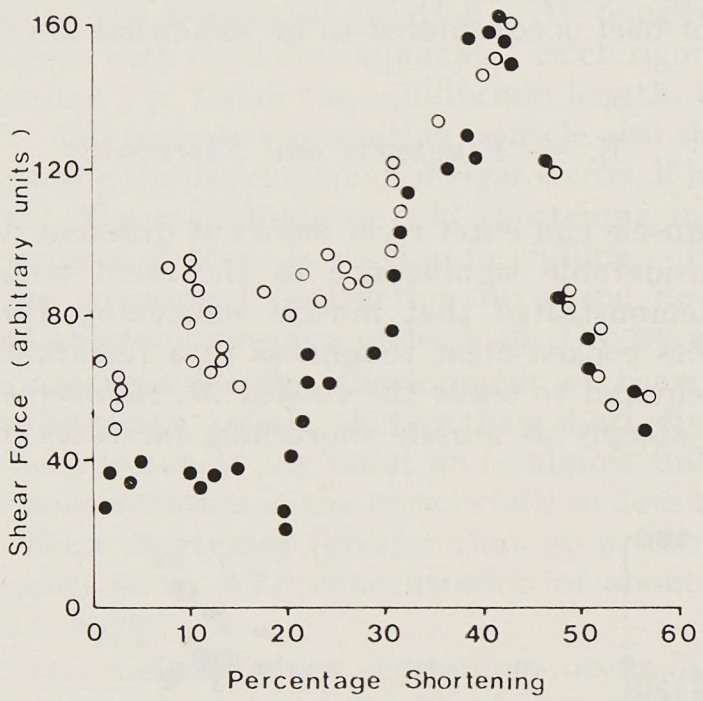

FIG. 2. — *Influence of pre-rigor muscle shortening on the extent of muscle aging, o, rigor muscle, stored at 15 °C for 24 hours post mortem; ●, aged muscle, stored at 15 °C for about 4 days post mortem* (after DAVEY *et al.*, 1967).

from about SF 65 to 25 over a period of 2.5 days, with a slight additional decline to SF 15 on storage for eight days. The shearing values of muscles shortened by 35 p. 100, in contrast, will similarly fall during 2.5 days storage at 15 °C but in this case from an initial value of SF 95 to a minimum of SF 75 (DAVEY *et al.*, 1967). Two signfiicant conclusions can be drawn from these studies. There is a limit to the tenderizing achieved by aging of M. *sternomandibularis*, and, shortening reduces the degree of tenderizing it is possible to achieve by aging.

The effect of maximal aging on the shortening-toughening relationship is shown in figure 2. It is stressed that the complete loss of tenderizing capacity at 40 p. 100 shortening shown in figure 2 is not a characteristic of all muscles

although high shortenings greatly reduce the tenderizing achieved by aging in those that have been studied (HERRING *et al.*, 1967).

Two quite clear processing requirements arise from our knowledge of rigor development, muscle shortenings and meat aging.

- Chilling or freezing of carcasses should take place after the completion of rigor development.
- Only then is maximal tenderizing of meat achieved through aging.

Processing specifications have been developed in New Zealand for both lamb and beef carcasses to overcome the hazards of rapid chilling and pre-rigor freezing. The specifications require that lamb carcasses or beef sides be conditioned, i.e. held at sufficiently high temperatures during rigor development to avoid toughening. The specifications additionally prescribe standards of hygiene and of humidity control to retard microbial growth. The extra tenderizing from aging forms part of the process (LOCKER *et al.*, 1975).

IV. — Electrical stimulation and rigor mortis

As an aid to energy, space and labour saving, it is important to the meat processor to be able to accelerate *post-mortem* changes in meat animals. It is possible to reduce rigor times by starvation and stress, but on humane grounds alone such procedures cannot be contemplated. It has been shown that by raising the temperature of freshly-slaughtered lambs to 45 °C, rigor develops in about 2 hours instead of the 12 to 16 hours if the carcasses were held at 10 °C (DAVEY and GILBERT, 1973). Some heat shortening occurs in those muscles that are not restrained by the skeleton. The development of rigor can also be achieved in about the same time by subjecting pre-rigor muscle to a brief period of very high pressure (BOUTON *et al.*, 1977). A more practical method is to subject carcasses of freshly-slaughtered animals to a brief period of electrical stimulation. The physiologist has often used electrical stimuli to develop early rigor in small muscles without having attempted to optimize the parameters to achieve a maximal rate of rigor development.

Electrical stimulation of freshly-slaughtered beef animals was first suggested by HARSHAM and DEATHERAGE (1951) as a means of inducing early aging in beef carcasses. INGRAM and INGRAM (1955) used the concept in a study of microbial growth rates in relation to rigor development in horse muscle. HALLUND and BENDALL (1965) showed that a brief period of *post-mortem* stimulation increased the glycolytic rate in pig muscle. DE FREMERY and POOL (1960), and more recently CARSE (1973), electrically stimulated chicken and lamb carcasses respectively with the specific aim of studying the relation between rates of *post-mortem* rigor development and meat quality.

Until cold shortening had been described and its importance in determining the toughness of meat fully recognized, the significance of the earlier investigations of HARSHAM and DEATHERAGE (1951) were not appreciated and their work lay dormant. Because of the earlier studies in New Zealand relating meat toughening to chilling and freezing practice, and because there is every good cause to reduce the pre-rigor phase in muscle from about 16 hours to a matter of a few hours, it was inevitable that the significance of electrical stimulation in overcoming un-

wanted shortening was recognized in New Zealand (CARSE, 1973). This study of CARSE has spawned a great deal of work both in New Zealand and around the world designed to increase the eating quality of meat through a brief period of carcass electrical stimulation. It emerges as a matter of good fortune that in addition to preserving acceptable tenderness electrical stimulation maintains a variety of other desirable meat properties, particularly colour (SAVELL, 1979).

A. — *Optimizing Stimulation Parameters*

Emphasizing the close similarity of responses to electrical stimulation by sheep and beef carcasses, this review has relied heavily on laboratory studies based on the *M. sternomandibularis* of beef animals, and the results have then been extended, not only to the whole beef side, but also to the lamb carcass.

Changes in time courses of pH fall are a convenient if indirect measure of the capacity of electrical stimulation to speed the development of *rigor mortis*

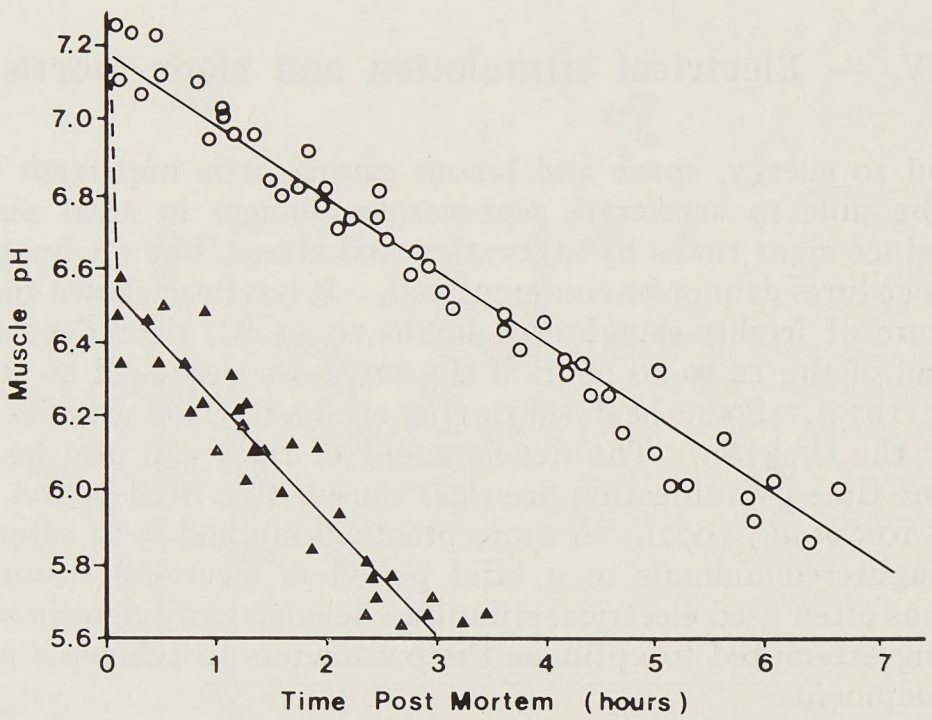

FIG. 3. — *Post mortem acceleration of pH in ox M. sternomandibularis muscles caused by electrical stimulation,* ○, *unstimulated control stored at 35 °C;* ●, *stimulated for one minute, then stored at 35 °C. Dotted vertical line represents ΔpH due to the stimulation treatment. Slope of regression lines represent* d*pH* /dt, *for each treatment* (courtesy of B. B. CHRYSTALL and C. E. DEVINE).

(fig. 3) (CHRYSTALL and DEVINE, 1978). The fall of pH occurs in two stages: A drop during the brief one to two minute period of stimulation (Δ pH), at a rate of approximately 0.4 pH units /minute: A further significant pH fall (0.5-1 units) at a rate (*d*pH /*dt*) of approximately 0.3 pH units /hour. Although this is only about 4 p. 100 of the rate occuring during stimulation it is still about twice as fast as that which occurs in unstimulated muscle. Regardless of refinements of the stimulation parameters *d*pH /*dt* is not increased beyond the two-fold rate of non-stimulated muscle. Electrical stimulation is not an essential requirement for increasing *d*pH /*dt*, since this can be made to occur merely by a period of vigorous muscle movement during slaughter.

It is the magnitude of ΔpH that largely determines the time delay before muscles lose their capacity to cold shorten. According to BENDALL (1975), this occurs once the muscle has fallen to approximately pH 6.0. As an example, the pH of non-stimulated beef *M. sternomandibularis* falls at about 0.1 pH units /hour taking approximately 7 hours to reach pH 6.0. In contrast, in optimally stimulated muscles, there is an immediate 0.6-0.7 pH fall (ΔpH) and a subsequent rate of fall (dpH /dt) of 0.3 pH units /hour, so that pH 6.0 is reached in about 1.5 hours. Recognizing therefore the merit in optimizing stimulation parameters, to what extent do variations in them and the manner in which they are applied affect the rate of onset of *rigor mortis* in beef and lamb?

B. — *Post-Mortem Delay and Muscle Temperature*

Conditions peculiar to each abattoir will dictate the time after slaughter at which it is convenient to stimulate carcasses. New Zealand experience shows however that there is a clear limit to this delay. If stimulation is undertaken within minutes after slaughter, quite low voltages and currents achieve large increases in rates of rigor onset, mainly through the triggering of a more rapid dpH /dt. Such stimulation occurs indirectly through a highly-active nervous system. With delay, even at high temperatures, the loss of neural responsiveness is very rapid and by 30 minutes in lamb, it is almost completely lost (CHRYSTALL *et al.*, 1980). Direct stimulation through the muscles then become important and voltages must be increased accordingly to achieve rapid onset of *rigor mortis* throughout the musculature. In normal processing the temperature of the surface of lamb carcasses, and to a lesser extent of beef carcasses falls quite quickly. A significant post-mortem delay in applying the stimulus then greatly reduces the ΔpH values obtained. Based on the temperature dependence of ΔpH, the energy of activation of the underlying biochemical change is 97 kJmol^{-1}, closely similar to that of the calcium-activated actomyosin ATPase of muscle. It is this presumably which largely gives rise to the ΔpH value. The energies of activation associated with dpH /dt in unstimulated and stimulated muscles are respectively 70 and 50 kJmol^{-1}. (CHRYSTALL and DEVINE, 1980). Obviously the rate limiting reaction which determines dpH /dt has changed due to stimulation. The practical outcomes from these observations are, that the rate-limiting reactions determining ΔpH and dpH /dt in stimulated muscles have high temperature coefficients and that their values fall markedly with a fall in carcass temperature.

C. — *Duration of Stimulus*

The early investigations in New Zealand (CARSE, 1973) used a stimulation period of 30 minutes and no special attempt was made to minimise the *post-mortem* delay. It was soon realized that such delay had a crucial role in determining stimulation effectiveness and it then became possible to examine more closely the importance of various stimulation parameters. Of these, the period of effective stimulation is of considerable practical importance. The two-fold increase in dpH /dt is prompted by a short burst of stimulation and it is only the value of ΔpH which increases progressively to the limit of 0.7 pH units by extending the stimulation time. A time of 2 minutes normally induced maximum ΔpH values. With lamb, but not with beef, a 2 minutes stimulation applied immedia-

tely following slaughter, tends to induce a degree of rigor stiffening before the carcasses have been fully dressed. For practical reasons therefore, a lesser period of stimulation is more appropriate in lamb to prevent such early stiffening.

D. — *Frequency and Polarity*

While continuous direct-current stimulation is possible, at voltages of less than 50 volts most procedures are based for many reasons on pulsed stimulation (CHRYSTALL and DEVINE, 1980). At a given voltage and period of stimulation, pulse frequency determines the magnitude of ΔpH. The value of ΔpH increases markedly to reach a broad peak with pulses between 5 and 17 per second. A 20 p. 100 fall in ΔpH from this peak occurs at higher frequencies. Since it is a simple task through electronic switching to achieve 14.28 pulses per second, New Zealand work has standardized on this alternating frequency to maximize responses and reduce energy inputs and unwanted polarization effects at the electrodes. Carcasses or sides stimulated at this frequency enter a tetanic contracture, the power of which, through the onset of fatigue, gradually reduces over a period of about 45 seconds.

E. — *Voltage and Current*

Most reported investigations refer to applied voltage, despite widely differing procedures for making electrical contact. The voltages should be of sufficient magnitude to overcome resistance effects at the electrodes and ensure a saturation flow of current through the musculature. Only then are maximal responses achieved throughout the carcass. Higher voltages ensure higher values of ΔpH up to a limit of 0.7 pH units (CHRYSTALL and DEVINE, 1978).

Such a simple analysis would seem to run contrary to the effective stimulation systems that have been described using voltages of less than 30 volts. Experience, especially with lambs, indicates that carcasses respond quite variously to a standard stimulation depending on their general physiological condition. There is every good reason therefore to overstate the voltage needed to ensure that values of ΔpH and $d\text{pH}/dt$ are as high as it is possible to achieve in all carcasses.

The voltage that is chosen is determined by a number of rather complex interacting factors and particularly aspects of the further processing of the carcass. In plants with small throughputs, supplying fresh meat to the local market, stimulation is useful in improving flavour, lean colour and in the case of beef marketing scores. Tenderness is of lesser importance since chilling is usually sufficiently slow that cold shortening is avoided and the meat can be aged successfully. Stimulation of beef is better performed by manual means in these smaller plants and to avoid expensive safety measures low voltages (< 30 v) can be contemplated.

At the other extreme, in large plants producing frozen meat for export, toughness development from cold and thaw shortenings is the overriding problem. High voltages are needed to overcome the contact resistance at rubbing electrode contacts which form the basis of simple automated systems, especially for stimulating lamb.

The meat must be close to or in *rigor mortis* before freezer entry to avoid cold shortening and more especially thaw shortening.

Whatever the final choice it is New Zealand experience that if toughness avoidance is the prime intention low voltage stimulation is not fully effective throughout the carcass (GILBERT and DAVEY — unpublished data). Maximal rates of pH fall are not even achieved at quite high voltages. Thus using 14.28 pulses per second for 1 minute applied between the fore and hind legs of beef carcass sides 400 v (RMS) induced 8hr pH values close to ultimate in *M. longissimus*, *M. semimembranosus*, and *M. triceps brachii*. In contrast this voltage did not by any means achieve the ultimates in such muscles as *M. rectus abdominus*, *M. brachiocephalicus* and *M. vastus lateralis*. To induce pH values near ultimate in 8hr in these muscles, a voltage of at least 800 v (RMS) was found to be required.

Operator safety is normally the controlling factor on which a final voltage choice is made. If this is less than ~ 30 V and is found to be sufficiently effective, then few safety measures are necessary. If a higher voltage is considered necessary then local regulation normally require a comprehensive set of reliable safety measures. High voltages ensuring current saturation through the musculature are then to be preferred since the cost of very high voltages is insignificant in relation to the advantage gained.

F. — *Chilling and Freezing of Stimulated Carcasses*

Once the pH of a muscle falls to approximately pH 6.0 it has virtually lost its capacity to cold shorten. However, if frozen at a pH that is only about 0.2 pH units above its ultimate, it will still actively thaw shorten (DAVEY and GILBERT, 1976). To achieve maximum dividends from stimulation, shortening, especially that associated with thaw rigor must be avoided. Built into New Zealand processing specifications for sheep meat is a post-stimulation delay before chiller or freezer entry to ensure that both cold and thaw shortenings are prevented. Lamb carcasses, because of their relatively small size, chill rapidly and $d\text{pH}/dt$ falls off markedly if there is no holding before freezing. Such carcasses will be toughened by a degree of cold shortening and, of more significance, by thaw shortening. The holding delay for lamb is 1.5 hours following stimulation at a temperature of not less than 6 °C.

V. — Toughness and electrical stimulation in processing practice

To what extent are cold and thaw shortenings significant in conventional meat processing practice? A clear demonstration has been obtained in large-scale studies undertaken to determine whether chilling or freezing of beef sides can be sufficiently rapid to toughen commercially important muscles. In one study (DAVEY, 1971) righthand sides of prime beef carcasses were slowly chilled to a deepleg temperature of 17 °C during 18 hours while the lefthand sides were rapidly chilled to 8 °C. The effects of the two chilling rates on the tenderness of the *M. longissimus* from the thoracic region is illustrated in figure 4. Rapid chilling toughened the muscle with some 80 p. 100 of the cooked meat being unacceptable (SF greater than 35). The toughened muscle did not tenderize to an acceptable level during aging as shown by the fact that the shearing forces of

half the samples remained above SF, 35. Very similar results have been obtained for muscles of young bulls, cows and calves, toughness in the main increasing with an increased rate of chilling (BUCTHER, 1972). More recently MARTIN *et al.* (1977), confirmed these studies during an examination of the effect of animal stress and glycolytic rate on toughness development in chilled, beef sides. They further demonstrated that in muscles that achieved early rigor, toughness and cold shortening were reduced.

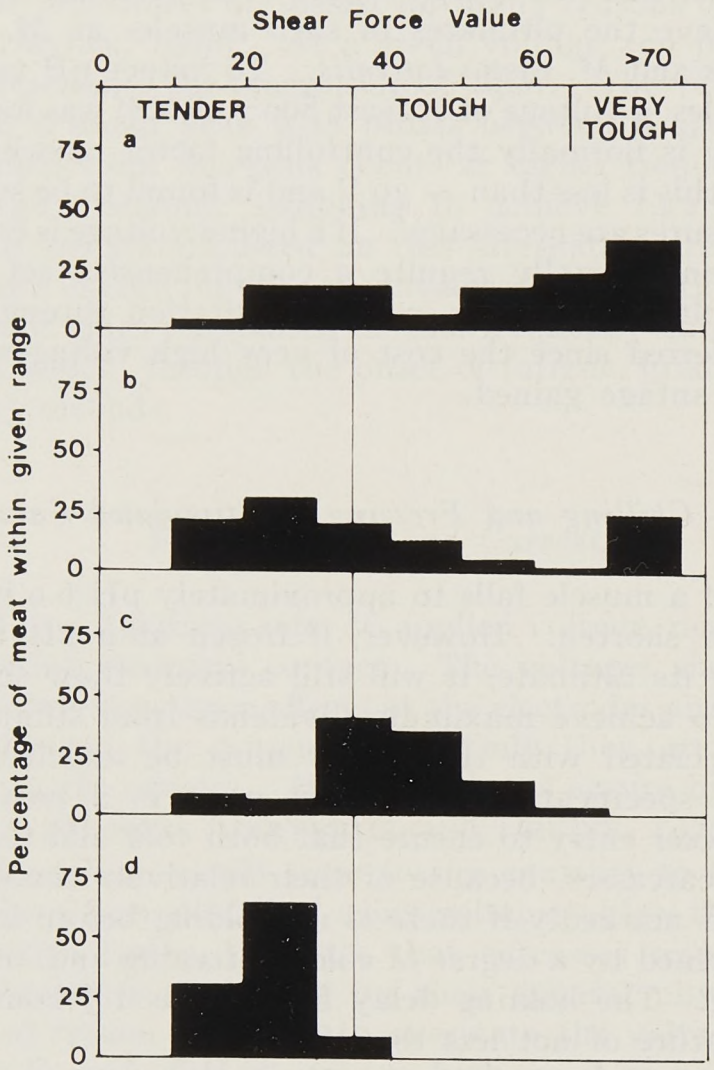

FIG. 4. — *The relationships between pre-rigor shortening and shearing force for cooked, rigor and aged beef M. longissimus. Muscles were excised at 18 hours post-mortem. Rigor samples* (a, c) *were then cooked and analyzed. Aged samples* (b, d) *were held for an additional 48 hours at 15 °C, then cooked and analyzed. Samples* a *and* b *were obtained from rapidly chilled sides. Samples* c *and* d *were obtained from relatively slowly chilled sides* (after DAVEY, 1971). *See Text.*

In New Zealand experience electrical stimulation has a prime role of overcoming the toughness of rapidly-chilled beef sides. In one major laboratory study the sides were subjected to 1-2 minutes of high voltage (3 600 V) electrical stimulation immediately after carcass dressing (DAVEY *et al.*, 1976). The time for rigor setting was reduced from 24 hours to about 5 hours. Even with rapid chilling, (deep leg muscle temperature ~ 8 °C at 24 hours) the stimulated carcasses were still warm at rigor entry, so that cold shortening did not develop. The meat was then reasonably tender and could be aged to a high degree of uniform tenderness (fig. 5). In other words, the tenderness of slowly chilled beef

can be similarly achieved with rapid chilling, so long as the sides have been electrically stimulated.

In an extension of this study (GILBERT and DAVEY, 1976) electrically stimulated beef sides were boned out at 5 hours since by then they had entered *rigor mortis*. Fast chilling during the early period hardened the surface fat and aided boning. As the meat was set in *rigor mortis* there was no risk of toughening from cold and thaw shortening in the boned out cuts. The eating quality of the meat

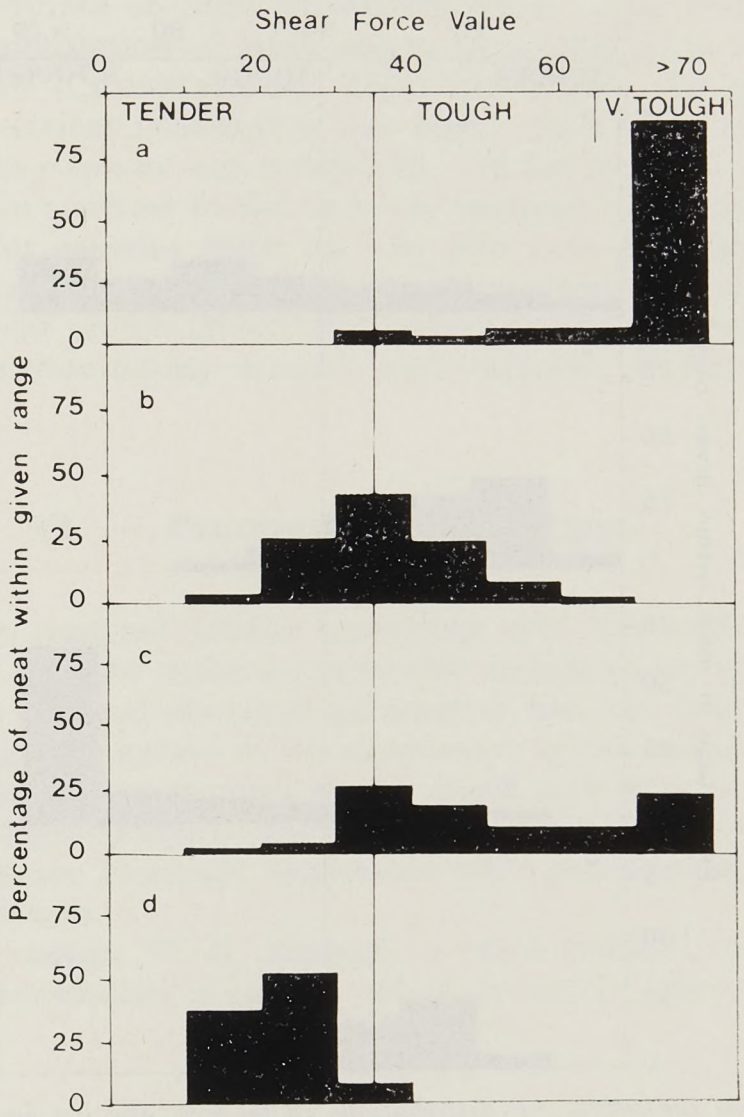

FIG. 5. — *Histograms indicating SF distributions in the M. longissimus of beef carcasses chilled at a rate sufficient to induce toughening through cold shortening. Muscles were excised at 18 hours post-mortem. Unstimulated rigor sample* a; *and aged sample* c. *Stimulated rigor sample* b, *and aged sample*, d. *The vertical line through SF, 35 is the demarcation between tender* (SF $<$ 35) *and tough* (SF $<$ 35) *beef.*

was as acceptable as unstimulated meat from slowly chilled sides boned out at 24 hours and tenderness was further improved by aging. There was no evidence of increased microbial spoilage from boning at 5 hours, while the meat was still warm. By boning carcasses earlier, chilling space and processing time is saved and subsequent handling of cartoned meat through either chilling or freezing can be carried out automatically.

Yet another variant is that of hot boning the sides of beef immediately following electrical stimulation. In one study (GILBERT *et al.*, 1977) the primal cuts were transferred to freezing or chilling at 2 hours *post mortem*. Despite

rapid freezing (7-10 hours) rigor was achieved before the meat temperature had fallen low enough (< 8 °C) to induce cold shortening. The meat was as tender as that boned from sides chilled conventionally for 24 hours. Alternatively, if the cuts were chilled to 5 °C over a period of 46 hours before freezing, shortening was not only avoided but the meat aged to a highly acceptable and uniform level. Although bacterial proliferation was limited by the much shorter processing time,

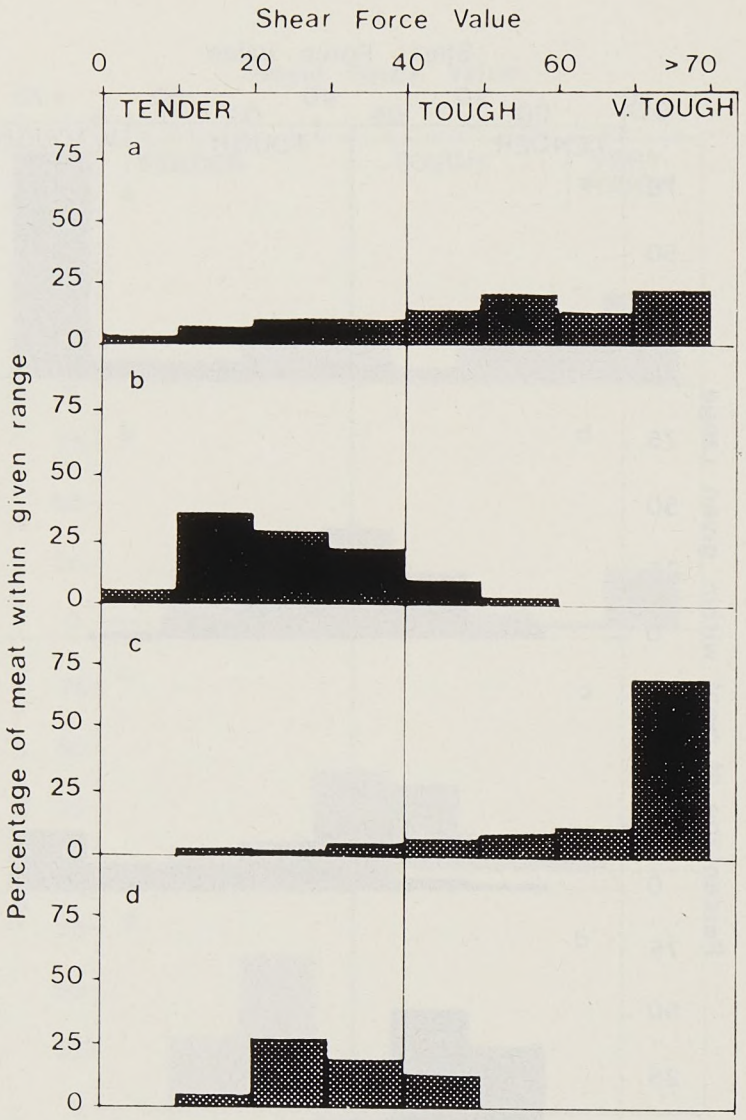

FIG. 6. — *Histograms indicating SF distributions in the loin and leg muscles of lamb carcasses frozen rapidly 2 hours after slaughter. Tenderness was measured on cuts roasted from the frozen state. The loin muscle analyzed was M. longissimus, and the leg muscles M. biceps femoris, M. semimembranosus, M. glutaeus medius, M. adductor and M. quadriceps femoris. Unstimulated loin and leg, respectively* (a *and* c). *Stimulated loin and leg respectively* (b *and* d). *The vertical line through SF 40 is the demarcation between tender* (SF < 40) *and tough* (SF < 40) *lamb.*

further studies will be necessary to determine whether selective microfloral growth occurs in the packaged, hot-boned meat.

Of more importance to New Zealand practice has been the development of practical processing strategies based on sheep meats.

The claim that both cold and thaw shortenings in lamb are matters for practical concern is justified in the paper of MARSH *et al.* (1968) describing the effect on loin muscle (*in situ M. longissimus*) tenderness of chilling and freezing lamb carcasses before the attainment of *rigor mortis*. The tenderness of the cooked

meat was greatly affected by the time-temperature patternof chilling and freezing. Carcasses frozen rapidly with little *post-mortem* delay were, on average, tough with about 50 p. 100 having a shearing force over SF 40. On the other hand, loins from lamb carcasses subjected to a delay (24 hours, 15-20 °C) and then slowly frozen were all very tender.

The impact of electrical stimulation technology on lamb processing has been considerable and since the report of CARSE (1973) much has been done to refine the technology to permit very rapid carcass throughputs (in New Zealand up to 50 carcasses per minute on some production lines). The first in-plant application of electrical stimulation of lamb was in 1974 with up to 20,000 carcasses per day being treated (CHRYSTALL and HAGYARD, 1976). At that stage high-voltage (3 600 V) electrical stimulation was used. As a guide to the procedure the pH in the *M. longissimus* was monitored. It fell to pH 6 or lower within 1 hour of slaughter, in contrast to the 14 hours required by unstimulated muscle. Shear-force values for muscles from leg and loin cuts of stimulated carcasses roasted from the frozen state were about half of those from unstimulated carcasses (fig. 6). A more recent review (HAGYARD, 1980) describes progress in using optimized conditions for stimulating dressed lamb carcasses in New Zealand.

VI. — Practical considérations

Since the earlier reported studies extending over the period 1973 to 1976 much work has been done to achieve practicable specifications for both beef and sheep animals. The original electrical parameters had not been optimized nor had an understanding been gained of the significance of the loss of carcass responsiveness to stimulation either through neural death or lowered carcass temperature. Very high voltages (3 600 V) and currents (2 amps peak) were found necessary largely to overcome electrode impedance with pelt-on stimulation, and to ensure current saturation.

As indicated in section IV E, although a much reduced voltage (< 30 V) can be used if the stimulation is carried out immediately after death, based on

TABLE 1

Requirements for the successful electrical stimulation of beef sides and lamb carcasses after dressing

	Beef	Lamb (*)
Time from slaughter	< 60 min	< 30 min
Stimulation time	⩾ 2 min	⩽ 1.5 min
Stimulation parameters		
Pulse repetition rate	14.28 /sec	14.28 /sec
Pulse width	10 msec	10 msec
Pulse voltage (peak) from 800 V (rms)	~ 1 130 V	~ 1 130 V

(*) There is also a specified delay (not shown) before the lamb carcasses can be frozen.

rates of pH fall, not all muscles are excited to the same extent if a single stimulation pathway between neck and foreleg electrodes is used. For this reason higher voltages are used in New Zealand. Although the stimulus can be applied at any stage during carcass dressing, there are good reasons for carrying it out after final carcass inspection. Table 1 summarizes critical parameters as used in New Zealand for sheep and beef animals after dressing. With certain adjustments specifications are also available for stimulation at any stage after slaughter. The requirements shown in the table are based on the optimized parameters and time-temperature requirements to achieve the required rates or *rigor mortis* development throughout the carcass. To achieve reliable stimulation of lamb, usually by automatic means, and at high rates of throughput, the prescribed conditions have to be met with little tolerance. With beef, considerable departures from the ideal parameters are possible without appreciable loss of meat quality. This is because beef carcasses retain their heat and are therefore less prone to cold shortening. However there are operational and quality assurance advantages in standardizing on a set of stimulation parameters which are, as far as possible, the same for sheep and cattle.

VII. — Future development

The avoidance of most of the toughening associated with rapid chilling and freezing is so simply achieved by carcass electrical stimulation, that alternative solutions would appear to be superfluous. Without the confusing intervention of such processing toughness, systematic attention can now be given to the alleviation of toughness related to such live-animal factors as breed, growth rate, maturity and stress.

Since, by good fortune, the early development of *rigor mortis* through electrical stimulation appears to improve a wide spectrum of other sought-after qualities more attention will no doubt be given to these. The muscles that make up a carcass all have their own special palatability characteristics and range from high grade grilling cuts to lower grade stewing cuts. Since the greater proportion of a carcass is made up of the latter, research will undoubtedly be directed to their upgrading. Electrical stimulation may well have a significant role to play in achieving this goal.

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Catabolisme de l'ATP pendant la stimulation électrique Influence du type métabolique et contractile

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Le but de la stimulation électrique des carcasses d'animaux de boucherie est de prévenir, lors de l'installation de la rigidité cadavérique, les contractures éventuellement provoquées par les pratiques technologiques de réfrigération et de congélation. Ces contractures engendrent en effet une augmentation parfois considérable de la dureté de la viande, qu'il s'agisse de la contracture au froid (« cold-shortening » des anglo-saxons) observée lors de la réfrigération rapide des carcasses peu de temps après l'abattage, ou de la contracture de décongélation qui survient lors de la décongélation de viandes congelées avant installation complète de la rigidité cadavérique (Bendall, 1973).

Pour prévenir le risque de contracture, il faut abaisser et éventuellement annuler le taux d'adénosine triphosphate (ATP), composé dont la dégradation au niveau de l'appareil myofibrillaire fournit l'énergie nécessaire au glissement relatif des myofilaments. En pratique, pour éviter la contracture au froid, il faut attendre un pH de l'ordre de 6,0, soit une diminution d'environ 50 à 80 p. 100 du taux d'ATP initial; pour prévenir de façon certaine la contracture de décongélation, il convient d'attendre la disparition complète de l'ATP avant de commencer la congélation (Bendall, 1980*b*). C'est le rôle de la stimulation électrique d'accélérer l'hydrolyse de l'ATP en particulier au niveau du système contractile et de précipiter ainsi l'épuisement de ce composé. L'hydrolyse de l'ATP entraîne un abaissement du pH musculaire, les vitesses des deux phénomènes étant proportionnelles tout au long de l'installation de la rigidité cadavérique (Bendall, 1973). Par contre la relation liant à chaque instant le pH et le taux instantané d'ATP (qui résulte de l'équilibre entre l'hydrolyse et la resynthèse simultanée de l'ATP) est plus complexe. Selon Bendall (1980*a*) elle est linéaire pendant la phase de diminution rapide du taux d'ATP (entre des taux d'ATP égaux à 90 p. 100 et 10 p. 100 du taux initial) et peut s'exprimer par la formule :

$$ATP/ATP_{10} = 1{,}63 - 1{,}7\,\frac{pHe - pH}{pHe - pHf}$$

où ATP_{10} = taux initial d'ATP (10 mn post mortem)
ATP, pH = taux d'ATP et pH à l'instant t
pHe = pH extrapolé au taux d'acide lactique nul
pHf = pH ultime (lorsque la rigidité cadavérique est installée)

La mesure de la vitesse de chute du pH permet donc d'apprécier indirectement le rythme de l'hydrolyse d'ATP dans le muscle *post-mortem*, de façon très précise si l'on connaît le facteur (variable entre muscles d'une même espèce animale et entre espèces) qui relie ces deux grandeurs. La première est beaucoup plus facile à mesurer que la seconde, ce qui explique que si de nombreux auteurs ont décrit l'évolution du pH musculaire lors de la stimulation électrique (Harsham et Deatherage; 1951; Carse, 1973; Chrystall et Hagyard, 1975; Davey *et al.*, 1975; Chrystall et Devine, 1978; Jonsson *et al.*, 1978; Bouton *et al.*, 1978) seuls Bendall et ses collaborateurs du M.R.I. de Langford (Grande-Bretagne) (Bendall, 1976; Bendall *et al.*, 1976; Bendall, 1980*b*) ont donné dans la littérature scientifique des résultats détaillés sur le catabolisme de l'ATP dans les muscles soumis à la stimulation. A l'I.N.R.A., Boucheteille (1978) puis Houlier (1979) ont étudié également l'hydrolyse de l'ATP et l'évolution du taux de ce dernier dans des muscles de lapin, de mouton et de bœuf soumis à la stimulation électrique. Aussi est-ce à ces auteurs que nous emprunterons l'essentiel des résultats que nous rapportons et discutons ici.

I. — Stimulation électrique et catabolisme de l'ATP

I.1. — *Rythme d'hydrolyse de l'ATP*

Bendall (1976) stimulait des lapins et des moutons, dans les 15 minutes suivant l'abattage, à l'aide d'une magnéto (6 Hz — 1 200 volts) ou d'un stimulateur délivrant un courant alternatif (250 V — 1 à 20 Hz). A 15 Hz, fréquence la plus couramment employée, l'intensité du courant atteignait 0,3 A dans les carcasses de lapin et 0,8 A dans celles de mouton.

TABLEAU I

Évolution du pH, *du potentiel de « Liaisons phosphate riches en énergie »* (~ P) *et du taux d'ATP dans les muscles* longissimus dorsi *de lapins et de moutons stimulés électriquement* (Bendall, 1976).

	Lapins		Moutons	
	Témoins	Stimulés 120 s	Témoins	Stimulés 120 s
Δ pH	— 0,05	— 0,32	— 0,02	— 0,42
Δ pH / Δt après stimulation	— 0,35	— 0,62	— 0,14	— 0,27
$\Delta \sim$ P	— 5,0	— 48,2	— 3,0	— 50,2
$\Delta \sim$ P / Δt après stimulation	— 37,0	80,0	14,3	27,5

Δ pH = chute de pH pendant la stimulation.
$\sim$ P = µmol. g^{-1}; Δ pH / Δt = unités pH.h^{-1}; $\Delta \sim$ P / Δt = µmol. g^{-1}. h^{-1}.
Conditions de stimulation : 250 V; 1 à 20 Hz.

Les résultats montrent que la vitesse d'hydrolyse de l'ATP ($\Delta \sim P / \Delta t$) est grandement accélérée pendant la stimulation, et que cette accélération se poursuit dans une certaine mesure après la fin de la stimulation : la vitesse d'hydrolyse reste environ deux fois plus élevée après la fin de la stimulation dans les muscles stimulés que dans les muscles témoins, après correction pour les différences de température.

Sur des carcasses de bovins soumises à une stimulation sous un voltage de 700 V, BENDALL *et al.* (1976) obtenaient des résultats comparables, à savoir une nette accélération du catabolisme de l'ATP et par suite de la chute du pH. Ils étudièrent de façon détaillée, dans le muscle *Triceps brachii*, l'effet du courant sur les phénomènes biochimiques pendant le cours même de la stimulation : ils purent ainsi montrer que le catabolisme de l'ATP est accéléré surtout pendant les 30 premières secondes; il décline ensuite relativement vite et retourne à sa valeur normale (avant stimulation) dès l'arrêt du passage du courant électrique.

TABLEAU 2

Évolution du pH et catabolisme de l'ATP dans le muscle triceps brachii *de bovin pendant la stimulation électrique* (700 v, 120 s) (BENDALL *et al.*, 1976).

Temps (s)	pH	$\Delta \sim P / \Delta t$	$\Delta pH / \Delta t$	$\Delta \sim P / \Delta pH$
0	7,15	5 220	66	95
30	6,60	1 190	14,4	99
60	6,48	610	7,2	102
90	6,42	610	7,2	102
120	6,36	17	—	—

$\Delta \sim P / \Delta t$: μmol. $g^{-1}.h^{-1}$ — Δ pH $/ \Delta t$: unités pH.h^{-1}.

Pendant les 15 premières secondes de stimulation, le taux d'hydrolyse de l'ATP approchait, selon BENDALL, le taux théorique attendu hors d'une tétanisation complète du muscle (10 000 μ mol.g^{-1}. h^{-1}). Ce résultat est à rapprocher de celui rapporté par CHRYSTALL et DEVINE (1980) selon qui l'énergie d'activation de la chute de pH pendant les 30 premières secondes d'une stimulation à 14,28 Hz (fréquence la plus efficace dans leurs conditions d'expérience) est identique à celle de l'énergie d'activation de l'actomyosine-ATPase, ce qui indique une activation maximale du système contractile du muscle. Cette énergie d'activation diminue ensuite avec l'épuisement du muscle, de même que la vitesse d'hydrolyse d'ATP rapportée par BENDALL.

Notons aussi que ces résultats nous permettent de vérifier la linéarité de la relation liant $\Delta \sim P$ et Δ pH : aux différentes périodes de la stimulation, une chute de pH d'une unité correspond à une consommation d'environ 100 μ mol.g^{-1} d'ATP (dernière colonne du tabl. 2).

Nous avons remarqué que selon BENDALL *et al.* (1976), chez le bœuf, l'accélération de l'hydrolyse de l'ATP, donc de la chute du pH, cesse avec la stimulation. Ceci contredit les observations de CHRYSTALL et DEVINE (1978) sur le muscle *Sternomandibularis* de bovin : ces auteurs observaient une poursuite de la chute du

pH à un rythme accéléré après la fin de la stimulation électrique. Bendall (1980 *b*) avance, comme explication à cette contradiction, une éventuelle détérioration des membranes cellulaires dans les conditions de relativement haut voltage appliquées par Chrystall et Devine (1978) à savoir 5 à 60 V/cm. L'effet d'accélération après stimulation ne serait d'ailleurs pas observé jusqu'à une valeur du champ de 60 V/cm lorsque le courant est appliqué perpendiculairement aux fibres musculaires (Bendall, 1980 *b*).

1.2. — *Évolution du taux d'ATP musculaire pendant et après la stimulation*

D'un point de vue pratique, le plus important est bien sûr la cinétique d'évolution du taux d'ATP dans le muscle : c'est ce taux qui conditionne en effet les possibilités de réfrigération et de congélation. Boucheteille (1978) a mesuré le taux d'ATP dans les muscles *Longissimus dorsi* et *Biceps femoris* du lapin pendant

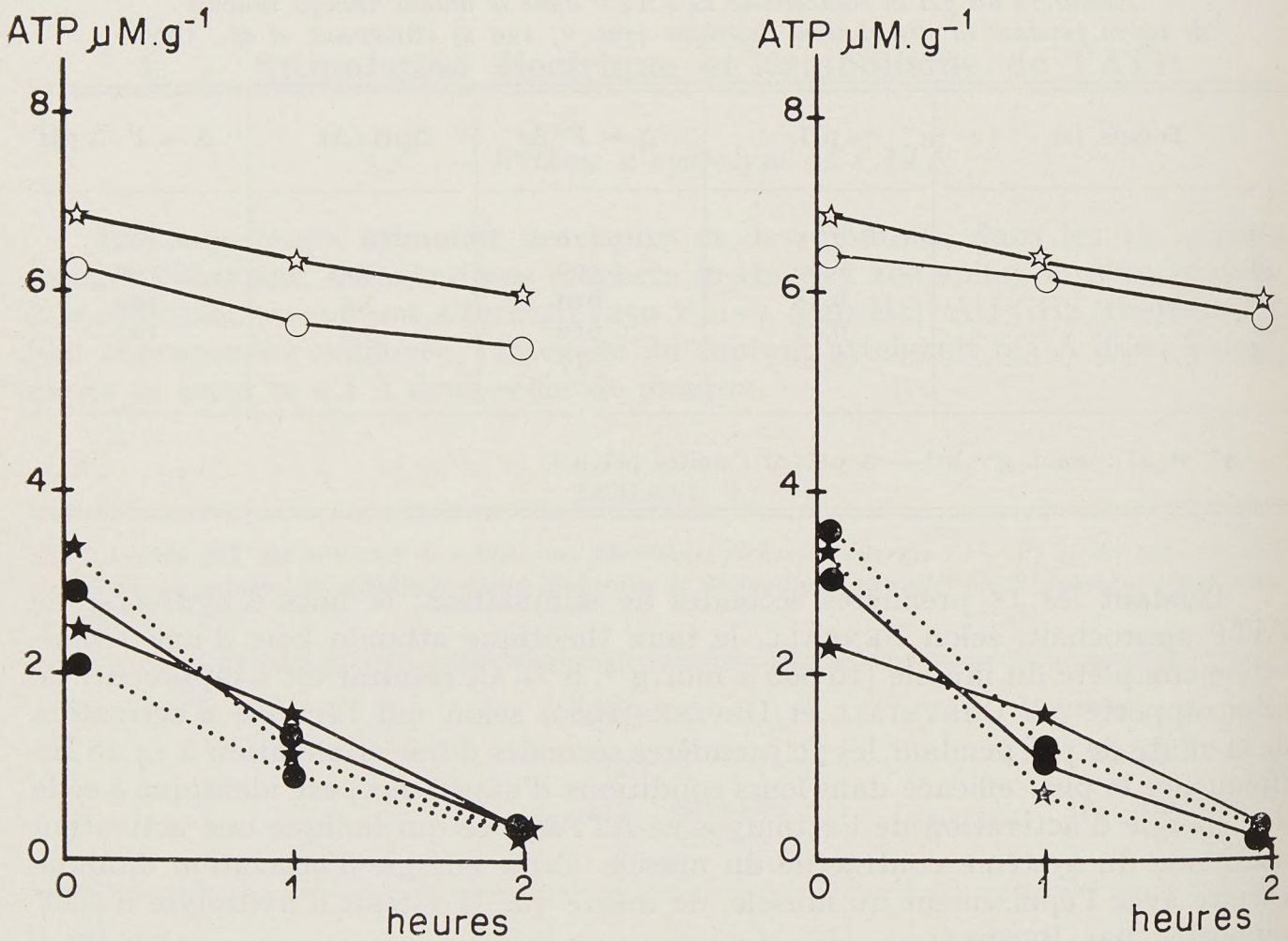

Fig. 1. — *Évolution du taux d'ATP dans les muscles* Longissimus Dorsi *et* Biceps Femoris *de carcasses de lapin stimulées électriquement* (120 s; 12,5 Hz).

Les étoiles correspondent au muscle *Biceps Femoris* et les cercles au muscle *Longissimus Dorsi ;* en blanc : animaux témoins, en noir : animaux stimulés.

Figure de gauche : Inversion de la polarité toutes les 5 secondes; en trait continu stimulation sous 100 volts, en pointillé stimulation sous 200 volts.

Figure de droite : Stimulation sous 100 volts avec inversion toutes les 5 secondes (trait plein) ou toutes les 30 secondes (en pointillé).

et après la stimulation électrique : ses résultats montrent que l'épuisement de l'ATP est réalisé environ 2 heures après la fin de la stimulation, alors que la teneur d'ATP avoisine encore 90 p. 100 du taux initial dans les muscles des animaux témoins (fig. 1). Ces résultats sont très comparables à ceux de BENDALL (1976).

BENDALL (1976, 1980 *b*) a déterminé les délais nécessaires à la diminution de moitié où à l'épuisement du taux d'ATP dans les muscles de bovins et d'ovins réfrigérés normalement ou après stimulation électrique (tabl. 3).

TABLEAU 3

Délais (en h) *nécessaires à la diminution de l'ATP à différents taux* (en p. 100 du taux initial) *dans les muscles* longissimus dorsi (LD) *et* biceps femoris (BF) *de carcasses de bovins et d'ovins stimulées électriquement* (BENDALL, 1976, 1980 *b*).

ATP p. 100 du taux initial	Bovins				Ovins			
	Témoins		Stimulés		Témoins		Stimulés	
	LD	BF	LD	BF	LD	BF	LD	BF
50	10,0	8,5	1,1	1,0	4,4	4,1	1,6	0,4
5	—	—	—	—	4,0	5,6	4,7	1,0
0	15,0	14,0	3,0	3,0	—	—	—	—

Conditions de stimulation : bovins = 12 minutes *post-mortem*, 680 V;
ovins = environ 15 minutes *post-mortem*, 250 V.

BENDALL (1980 *b*) conclut de ces résultats que l'on peut commencer en toute sécurité la réfrigération des carcasses environ 2 heures après l'abattage, mais qu'il est prudent d'attendre 6 heures pour débuter la congélation. Dans ce dernier cas en effet, il faut que toute trace d'ATP ait disparu pour être certain d'éviter la rigor de décongélation, si néfaste à la tendreté.

Comme nous l'avons noté plus haut, beaucoup d'autres chercheurs ont évalué la vitesse de chute du pH dans des conditions de stimulation très variées : carcasses habillées ou non, entières ou fendues; hauts ou bas voltages; formes et durées d'impulsions diverses... L'examen de leurs résultats aboutit à des conclusions similaires à celles que BENDALL et ses collaborateurs tiraient de leurs propres expérimentations : l'efficacité de la stimulation augmente avec le voltage (dans certaines limites, ainsi l'efficacité maximale est atteinte vers 600-700 V dans les carcasses de bovins); la durée des impulsions a peu d'importance, bien que des impulsions très courtes soient moins efficaces; la fréquence optimale se situe vers 10 à 20 Hz. Nous n'insisterons pas sur ces points qui font l'objet d'un autre exposé au cours de cette réunion (cf. DAVEY et CHRYSTALL p. 547).

II. — Hétérogénéité de la réponse à la stimulation dans la carcasse

Une revue des résultats obtenus par les divers auteurs qui ont évalué la chute du pH, au cours de la stimulation électrique, dans plusieurs muscles de la carcasse, révèle une grande hétérogénéité dans la réponse des différents muscles, particulièrement lorsque la stimulation est réalisée à bas voltage. Nous rapportons dans le tableau 4 les résultats obtenus par BOUCHETEILLE (1978) lors de la stimulation d'une carcasse de bovin sous 560 volts.

TABLEAU 4

pH mesuré deux heures post -mortem *dans divers muscles de la carcasse de bovin stimulée électriquement* (560 V; 12,5 Hz; durée de la stimulation = 120 s; inversion de la polarité toutes les 5 secondes; stimulation 5 mn après la saignée).

Épaule		Tronc		Cuisse	
Muscle	pH	Muscle	pH	Muscle	pH
Infraspinatus	6,57	*Longissimus dorsi* . .	5,52	*S. membranosus* . . .	5,60
Supraspinatus. . . .	6,60	*Rectus abdominis* . .	5,95	*S. tendinosus*	5,52
Subscapularis	6,25	*Diaphragma*	6,32	*Gluteus medius* . . .	5,70
Triceps brachii . . .	6,15	*Pectoralis profundus* .	5,52	*T. fasciae latae* . . .	5,75
		Psoas major	5,57	*Biceps femoris* . . .	5,55

La première raison que l'on peut invoquer pour expliquer une telle hétérogénéité consiste dans la non-uniformité du champ électrique à travers la carcasse au cours de la stimulation. Il n'y a à notre connaissance aucun résultat relatif à ce problème dans la littérature, si ce n'est ceux rapportés par BENDALL (1980 *b*) pour la carcasse de bovin : dans des carcasses stimulées à partir des muscles du cou et des tendons d'Achille sous une tension de 600 volts, BENDALL observait une chute de tension de 2,2 V /cm dans le *Longissimus dorsi* ((partie lombaire) contre 0,4 V /cm dans le *Triceps brachii*, pour une chute moyenne de 3 V /cm entre les deux électrodes. HOULIER et SALE (1980) à l'I.N.R.A. ont étudié la répartition du champ électrique dans la carcasse de mouton, stimulée à partir du cou et des tendons d'Achille après habillage, environ 20 mn *post-mortem :* leurs résultats démontrent également une très grande hétérogénéité du champ électrique. Cependans cette hétérogénéité ne peut pas expliquer toutes les variations observées dans l'efficacité de la stimulation (tabl. 5).

A partir de ses observations sur le lapin et le bovin, BOUCHETEILLE (1978) faisait l'hypothèse que des variations du type métabolique musculaire pourraient contribuer aux différences inter-muscles de réponse à la stimulation : il notait que les muscles blancs se contractaient à des voltages plus faibles que les muscles rouges, et montraient une réponse plus uniforme dans des conditions variées de stimulation (voltage, fréquence). SWATLAND (1975) observait que lors de la sti-

TABLEAU 5

Champ électrique, chute du pH et diminution du potentiel de « liaisons phosphate riches en énergie » au cours de la stimulation électrique dans quelques muscles de la carcasse de mouton stimulée électriquement (600 V; 12,5 Hz; durée totale 180 s).

Muscle	Champ (V/cm)	Δ pH	Δ ~ P μ mol^{-1}
Triceps brachii (caput longum)	1,5	0,72	— 55
Supraspinatus	1,4	0,57	— 36
Flexor digitorum superficialis	5,5	0,69	— 40

mulation de muscle de bovin isolé, les fibres à faible teneur en mitochondries étaient le siège d'une glycolyse plus intense que celle qui prenait place dans les fibres riches en mitochondries. HOULIER *et al.* (1980) ont précisé ces premières observations en montrant que chez le mouton l'efficacité de la stimulation, au niveau du muscle entier, est largement dépendante du type métabolique et contractile prédominant dans le muscle. Ils ont évalué la réponse à la stimulation de trois muscles de type métabolique et contractile différent — le *Longissimus dorsi* (LD) à prédominance rouge-rapide, le *Tensor fasciae latae* (TFL) à prédominance blanche-rapide et le *Supraspinatus* (SS) à prédominance rouge-lente, (voir tabl. 6), à l'aide d'un dispositif permettant de contrôler exactement la valeur du champ électrique.

TABLEAU 6

Caractéristiques métaboliques et contractiles de 3 muscles de mouton

Muscle	ATPase myofibrillaire μ eq KOH.mn^{-1}.mg^{-1}	Déshydrogenase lactique UI.(g proteine)$^{-1}$	Fer héminique μg.(g tissu frais)$^{-1}$
TFL.	0,26 ± 0,03	2 120 ± 183	5,5 ± 0,2
LD	0,24 ± 0,02	2 930 ± 225	10,9 ± 0,4
SS	0,18 ± 0,03	692 ± 110	10,3 ± 0,3

TFL : Tensor Fasciae Latae; LD : Longissimus dorsi; SS : Supraspinatus.

En faisant varier le champ électrique et la durée de stimulation, HOULIER et ses collaborateurs trouvèrent que pour des champs électriques inférieurs à 2 V/cm, l'influence du type contractile musculaire est très importante : la stimulation est très efficace pour le blanc-rapide TFL, un peu moins pour le rouge-rapide LD et est faible pour le rouge-lent SS. Ces différences s'atténuent mais ne s'annulent pas lorsque le champ électrique dépasse 2 V/cm, voltage à partir duquel l'efficacité de la stimulation plafonne. Le type contractile paraît beaucoup plus important que le type métabolique, comme on peut le voir sur la figure 2.

Nous ne disposons pas de résultats comparables pour les muscles de bovins Cependant nous avons pu porter sur un diagramme, les valeurs de chute du pH rapportées par BOUCHETEILLE (1978) contre les activités ATPasiques de l'actomyosine des muscles homologues récemment mesurées dans notre laboratoire. Bien que ces résultats, obtenus sur des animaux différents, méritent d'être vérifiés, il apparaît une assez bonne relation entre les deux grandeurs (fig. 3). L'efficacité très faible de la stimulation dans certains muscles de l'épaule s'explique probablement à la fois par leur faible activité ATPasique mais aussi par le faible champ électrique à leur niveau (cf. résultats de BENDALL, 1980, rapportés p. 568).

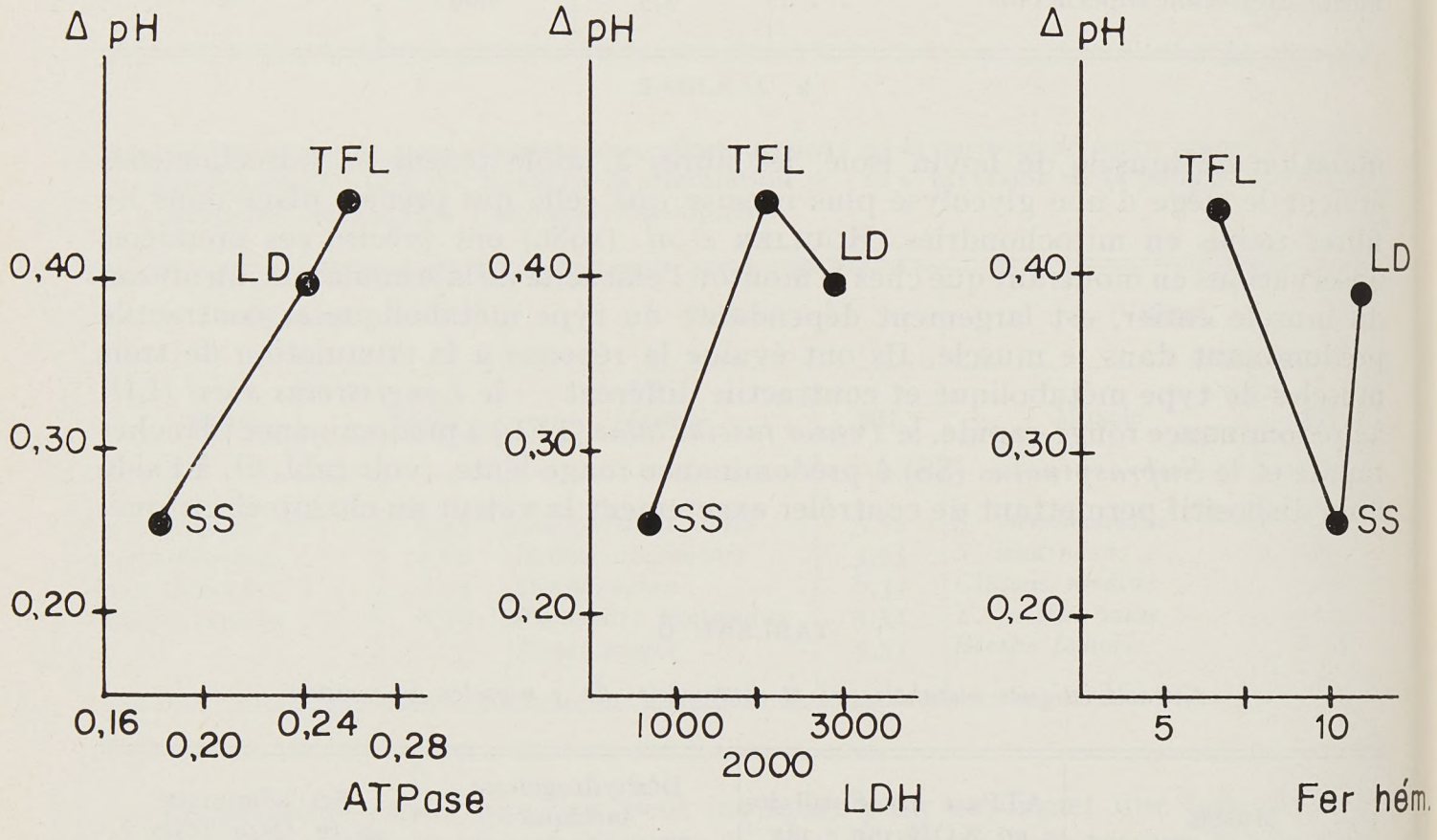

FIG. 2. — *Relation entre la chute du pH pendant la stimulation et les caractéristiques contractiles et métaboliques des muscles du mouton*

ATPase : μ eq.KOH.mg^{-1}. mn^{-1}; LDH : UI (g protéine)$^{-1}$; fer héminique : μg (g tissu frais)$^{-1}$. Stimulation pendant 120 s à 1 V/cm et 12,5 Hz.

L'efficacité de la stimulation apparaît donc directement liée à l'activité ATPasique myofibrillaire. Ceci pourrait résulter du fait que les myofibres à contraction rapide qui possèdent l'activité ATPasique myofibrillaire la plus élevée, sont innervées par des axones possédant un seuil d'activation plus bas (CLOSE, 1972, cité par SWATLAND, 1977), puisqu'il paraît bien établi que le courant électrique stimule la musculature par l'intermédiaire des terminaisons nerveuses présentes dans les muscles (BENDALL, 1980 *b*). Quoiqu'il en soit, ces observations apportent une explication aux résultats de BENDALL (1976, 1980 *b*) selon qui la variabilité intermuscle d'efficacité de la stimulation décroît lorsque le voltage appliqué à la carcasse augmente. Elles montrent que le type contractile musculaire pourrait constituer un facteur limitant très important dans la mise en œuvre de la stimulation à basse tension, dans laquelle la chute de tension moyenne dans l'ensemble de la carcasse se situe entre 0,3 et 1 V/cm. Elles indiquent également qu'il est important d'assu-

rer une répartition optimale du champ électrique dans la carcasse, par une localisation judicieuse des électrodes et une adaptation de la tension appliquée, si l'on veut obtenir une efficacité maximale de la stimulation dans toutes les parties de la carcasse.

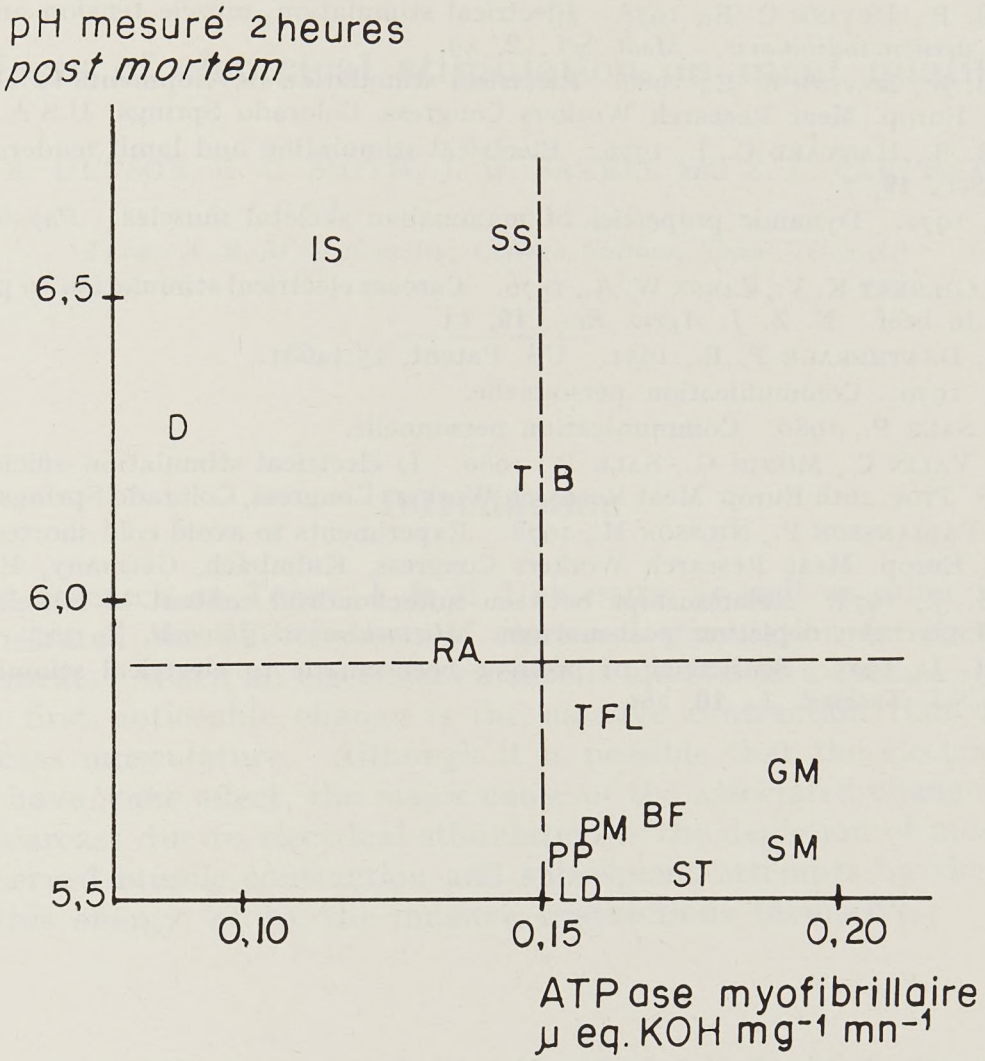

FIG. 3. — *Relation entre l'activité* ATPasique *myofibrillaire et la chute du* pH *dans quelques muscles de la carcasse de bovin stimulée électriquement* (mêmes conditions de stimulation qu'au tabl. 4).

BF : *Biceps femoris* — D : *Diaphragma* — GM : *Gluteus medius* — IS : *Infraspinatus* — LD : *Longissimus dorsi* — PP : *Pectoralis profundus* — PM : *Psoas major* — RA : *Rectus abdominis* — SM : *Semimembranosus* — ST : *Semitendinosus* — SS : *Supraspinatus* — TB : *Triceps brachii* — TFL : *Tensor fasciae latae.*

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Effects of electrical stimulation on meat quality *

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Introduction

Recent research at Texas A & M University as well as other institutions has demonstrated the effectiveness of electrical stimulation for increasing the quality of meat. When an electrical current is applied to a carcass shortly after death, the first noticeable change is the massive contraction that takes place in the carcass musculature. Although it is possible that the electrical current itself may have some effect, the major cause of the associated changes that take place in a carcass due to electrical stimulation is the depletion of muscle energy by the observed muscle contraction and subsequent attempts by the muscle to replenish this energy, or by the massive contractions themselves.

Accelerated post mortem glycolysis and rigor mortis

The attempts of the muscle to replenish the ATP used during electrical stimulation contractions, induce an acceleration of *post mortem* glycolysis and result in a rapid accumulation of lactic acid and a drop in pH (CARSE, 1973; BENDALL, 1976). In some studies a pH below 6.0 has been noted as early as one hour post-stimulation (BENDAL), 1976; CHRYSTALL *et al.*, 1980). The alteration in pH itself may have an effect on tenderness by causing alterations in the various muscle proteins, but, more likely, the lowered pH produces an environment conducive for other changes in the muscle proteins. A low pH and *rigor mortis* formation occurs before heat can be removed from the carcass, resulting in *rigor mortis* at a higher temperature (GEORGE *et al.*, 1980). The high temperature-low pH combination would also have an accelerating effect on acid proteases (DUTSON *et al.*, 1977). Development of *rigor mortis* at elevated temperatures has been associated with marked increases in tenderness (LOCKER and DAINES, 1976; DUTSON *et al.*, 1977) and could be a major factor in tenderness changes of electrically stimulated muscle.

The lowered pH also produces a more rapid development of a bright, more

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youthful lean color, a more even color over the whole muscle and slightly firmer lean, particularly when muscle is evaluated at early times *post mortem* (Cross *et al.*, 1979; McKeith *et al.*, 1980*a*, 1980*b*, 1980*c*; Savell *et al.*, 1978*c*, 1978*b*, 1979; Smith *et al.*, 1979*a*, 1979*b*). Veal and calf carcasses, as presented in Table 1, show a particularly marked improvement in color, texture and firmness when electrically stimulated (Savell *et al.*, 1980).

TABLE 1

Summary of effects of electrical stimulation of veal carcasses

Trait	Number of carcasses or samples	Percentage improvement (%)
Lean maturity score	40	3
Lean color score	80	12
Lean texture score	40	28
Lean firmness score	40	36

Adapted from Smith *et al.*, 1980.

Tenderness is one of the most important factors in acceptability of meat. Electrical stimulation has been demonstrated in numerous studies to increase meat tenderness. Studies at Texas A & M University, summary presented in Table 2, show that electrical stimulation improves the tenderness of beef by an average of 26 p. 100 as measured by a sensory panel and 23 p. 100 as measured by shear force analysis (Cross *et al.*, 1979; McKeith *et al.*, 1980*a*, 1980*c*; Savell *et al.*, 1977, 1978*a*, 1978*b*, 1978*c*, 1979; Seideman *et al.*, 1979; Smith *et al.*, 1977, 1979*a*, 1979*b*, 1980). In these experiments, the cattle which would have been most unacceptable in tenderness if not treated, had the greatest response to the electrical stimulation treatment. This has the effect of reducing the variability in tenderness of any population of cattle, in addition to improving their tenderness. The rate at which beef is tenderized during *post mortem* conditioning (aging) is also increased by electrical stimulation (Smith *et al.*, 1980). These studies show that electrically stimulated beef is more tender at two days *post mortem* than it would have been if not stimulated and aged fourteen days. Smith *et al.* (1980) also showed that by holding electrically stimulated beef for fourteen days *post mortem* it was fifteen percent more tender than it would have been if held for only two days *post mortem*. These studies demonstrate that electrical stimulation not only accelerates the *post mortem* aging process, but that *post mortem* aging in combination with electrical stimulation has a complementary effect on tenderness of beef.

The flavor of beef, as determined by sensory panel analysis (Table 2), has also been shown to be slightly improved (6 p. 100 increase) by electrical stimulation (McKeith *et al.*, 1980*a*, 1980*c*; Savell *et al.*, 1977, 1978*a*, 1978*b*, 1978*c*, 1979; Seideman *et al.*, 1979; Smith *et al.*, 1979*a*, 1979*b*, 1980). The flavor improvement is probably associated with the increased rate of *post mortem* biochemical reactions that are initiated by the electrical current and muscle contractions.

TABLE 2

Summary of effects of electrical stimulation of beef carcasses

Trait	Number of carcasses or samples	Percentage improvement (%)
Tenderness rating	452	26
Shear force value	656	23
Flavor rating	349	6
Lean maturity score	1,261	23
Lean color score	1,261	14
"Heat-ring" score	1,177	23
Firmness score	458	4
Marbling score	1,251	11
USDA quality grade	1,086	8

Adapted from SMITH *et al.*, 1980.

One study (HALL *et al.*, 1980) has demonstrated that the appearance of electrically stimulated round steaks displayed in a retail case maintained an acceptable bright color for one half to one day longer than did non stimulated control steaks. However, this study also showed that the retail appearance of ground beef was not improved by electrical stimulation.

The changes in meat caused by electrical stimulation are most likely produced by the increase in the rate of *post mortem* events such as pH decline and rigor formation. The events of rapid pH drop and rapid rigor formation of electrically stimulated muscle may not, in themselves, produce the changes, but they create the proper conditions for the changes to occur.

Cold shortening

Reports in the literature have indicated that electrical stimulation causes an increase in tenderness by preventing the toughening associated with cold shortening or thaw rigor (BENDALL *et al.*, 1976; CHRYSTALL and HAGYARD, 1975, 1976; DAVEY *et al.*, 1976). Measurement of the amount of shortening in electrically stimulated muscle by determination of sarcomere length has revealed that differences in shortening between electrically stimulated and non-stimulated sides of beef do not exist in most cases (SMITH *et al.*, 1977, 1979*a*; SAVELL *et al.*, 1977, 1979; MCKEITH *et al.*, 1979; SEIDEMAN *et al.*, 1979; WILL *et al.*, 1980; GEORGE *et al.*, 1980). However, differences in sarcomere length have been noted occasionally between electrically stimulated and non-stimulated muscle (SMITH *et al.*, 1977, 1979). GEORGE *et al.* (1980) found no difference in sarcomere length of stimulated and non-stimulated samples when using the laser technique of sarcomere measurement but did find differences when sarcomeres were measured on isolated fibers using the light microscope. Normally these two techniques produce identical sarcomere measurements (CROSS *et al.*, 1980). Sarcomere length values

TABLE 3

Sarcomere length values for various electrical stimulation experiment [1]

ES	NS	Level of probability	Study
1.83	1.84	NS	SAVELL *et al.* (1977)
1.73	1.75	NS	SAVELL *et al.* (1978*b*)
1.86	1.86	NS	SAVELL *et al.* (1979)
1.67	1.65	NS	BOWLING *et al.* (1978)
1.67	1.68	NS	BOWLING *et al.* (1978)
1.72	1.71	NS	BOWLING *et al.* (1978)
1.82	1.86	NS	SMITH *et al.* (1979*a*)
1.73	1.77	NS	SMITH *et al.* (1979*a*)
1.74	1.74	NS	SEIDEMAN *et al.* (1979)
1.96	1.84	$P < 0.05$	SMITH *et al.* (1979*b*)
1.58	1.58	NS	MCKEITH *et al.* (1979)
1.65	1.70	NS	WILL *et al.* (1979)
3.44	3.22	NS	WILL *et al.* (1979)
1.65	1.76	NS	WILL *et al.* (1979)
2.03	1.90	NS	WILL *et al.* (1979)
1.60	1.58	NS	GEORGE *et al.* (1980)
1.85	1.87	NS	GEORGE *et al.* (1980)
1.60	1.46	NS	GEORGE *et al.* (1980)
2.09	1.80	$P < 0.01$	GEORGE *et al.* (1980)

[1] From DUTSON *et al.* (1980*b*).

from various studies are presented in Table 3; results indicate that only two out of seventeen studies showed a significant effect ($P < 0.05$) of electrical stimulation on sarcomere length.

It appears from the number of studies that have been conducted and reported by DUTSON *et al.* (1980*b*), that electrical stimulation does not normally produce different sarcomere lengths than would have been present without the stimulation treatment. Thus, under normal chilling conditions, reduction of cold shortening is probably not a factor in the tenderness improvement caused by electrical stimulation. However, in some instances such as extreme chilling or in localized positions within a muscle, changes in sarcomere length may be prevented by electrical stimulation.

Proteolysis

Proteolysis has long been considered a possible mechanism for many of the tenderness changes that occur in *post mortem* muscle but the actual proof of this phenomenon has been elusive. Even so, studies have shown that conditions which favor proteolysis, such as acid pH and high temperature, also cause changes to take place that are associated with tender muscle and actually produce increased tenderness in some cases (DUTSON *et al.*, 1977; LOCKER and DAINES, 1976).

Electrical stimulation has been shown by many researchers to produce a low pH condition in the muscle while the temperature remains fairly high (DUTSON *et al.*, 1977; BENDALL, 1976; CHRYSTALL *et al.*, 1980; GEORGE *et al.*, 1980). This condition, in addition to producing tenderness-associated changes, has also been shown to cause a release of lysosomal enzymes from the membrane-bound condition into a milieu favorable for their action (MOELLER *et al.*, 1976, 1977).

Investigations were conducted in our laboratory on ovine muscle to determine if electrical stimulation also caused a release of enzymes from the lysosome. The data presented in Table 4 (DUTSON *et al.*, 1980*b*) indicates that a 24 p. 100 to 30 p. 100 increase in free activity of lysosomal enzymes is caused by electrical stimulation. At this point it is not certain if it is the electrical impulse itself or the lowered pH-high temperature that causes a rupturing of the lysosomal membrane. The release of these enzymes at an acid pH may be responsible for some of the tenderness change caused by electrical stimulation.

TABLE 4

Free activity changes in lysosomal enzyme caused by electrical stimulation (1)

Enzyme	Free activity increase (%)
β-glucuronidase	24
Cathepsin-C	30

(1) Adapted from DUTSON *et al.* (1980*a*).

Research by SAVELL *et al.* (1978*b*), NILSSON *et al.* (1979) and GEORGE *et al.* (1980) has shown that electrical stimulation causes an acceleration in the aging process of beef. If the aging process results from or is enhanced by proteolytic enzymes, then an increase in the aging process by electrical stimulation is further evidence that proteolysis may be activated by electrical stimulation.

Structural alterations

Research reported by SAVELL *et al.* (1978*c*) was the first to demonstrate that structural alterations occurred in muscles of animals that had been electrically stimulated. These alterations were demonstrated using both the light microscope and the electron microscope and appeared as contracture bands or contracture nodes.

Electron micrographs of electrically stimulated and non-stimulated muscle are presented in Figures 1 and 2, respectively. These micrographs show the characteristic differences between stimulated and non-stimulated muscle.

Disappearance of the banding pattern and disruption of the Z line is clearly evident in the contracture band area of the stimulated muscle. Disruption of the sarcoplasmic reticulum and slight separations of the myofibrils are also evident in some areas. Many of the alterations in the stimulated muscles appear very much like those that are caused in muscles having rapid *post mortem* glycolytic rates (DUTSON *et al.*, 1974; ABBOTT *et al.*, 1977), except for the contracture bands.

More recently, WILL *et al.* (1980) and GEORGE *et al.* (1980) have made similar observations of structural alterations in electrically stimulated muscle. These

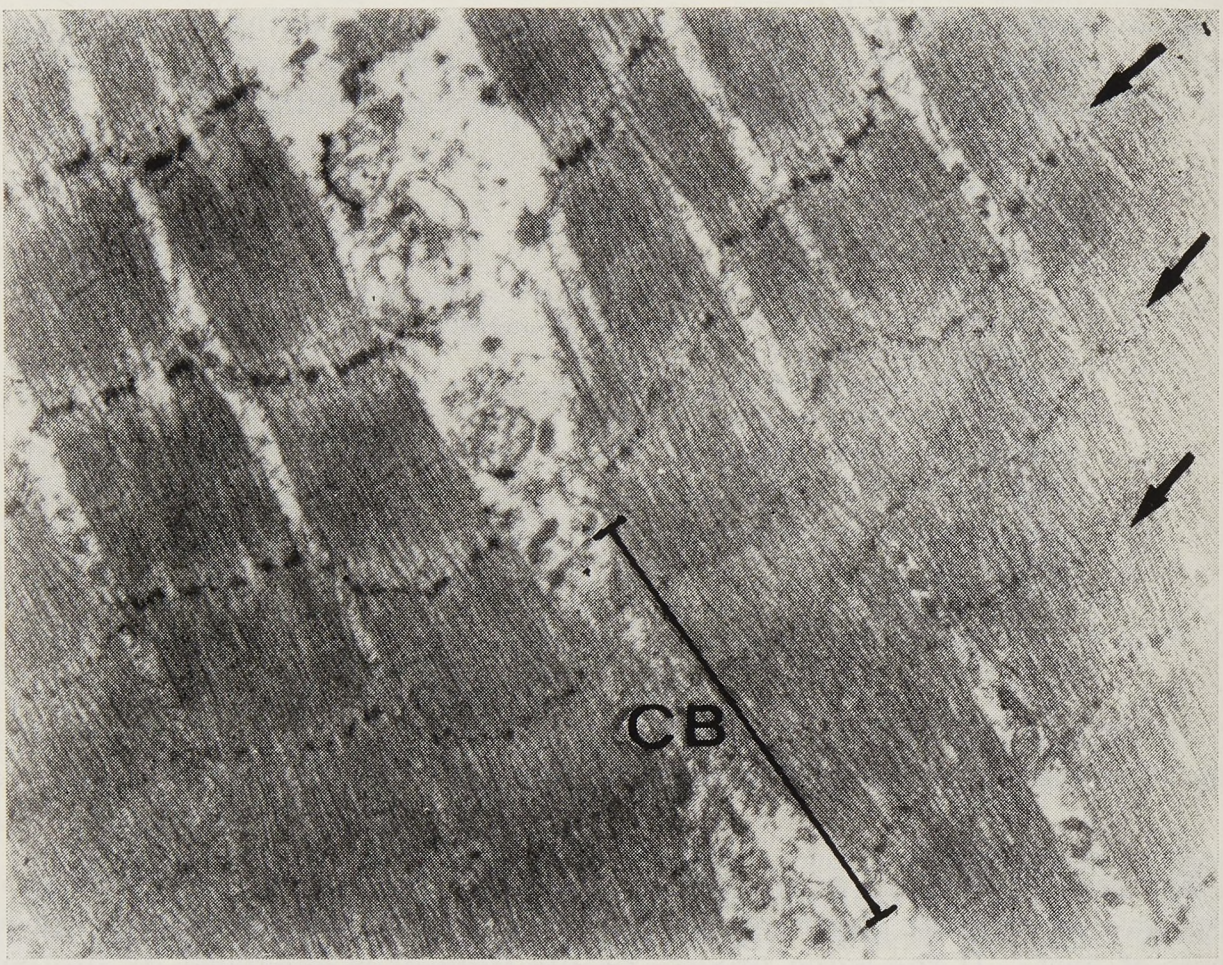

FIG. 1. — *Electron micrograph of electrically stimulated muscle immobilized and embedded at 6 hours* post mortem. *Arrows denote a continuation of Z line remnants. CB = contracture band area.*

authors both observed the structures we have termed contracture bands, but GEORGE *et al.* (1980) attributed these structures to precipitation of other protein material on the myofibrils. It is clearly evident from Figures 1 and 2 that the structures observed as contracture bands are continuous with the unaffected myofibrils and are derived from them. It is also evident that myofilaments and remnants of Z lines (arrows) are present in the contracture band and are continuous with their more normal counterparts in the unaffected portion of this micrograph. Thus, it is felt that the major portion of the structural abnormality in the contracture band area is due to extreme shortening of the sarcomeres and not due to precipitation of sarcoplasmic protein as described by GEORGE *et al.* (1980).

Chilling efficiency

Several packing plants in the U.S.A. have noted that the time at which the chill coolers reached a constant temperature was increased markedly after installation of an electrical stimulation unit. In order to test this observation, temperatures of stimulated and non-stimulated sides of the same carcass were accurately monitored under laboratory conditions (DUTSON, 1980; SCHNEIDER,

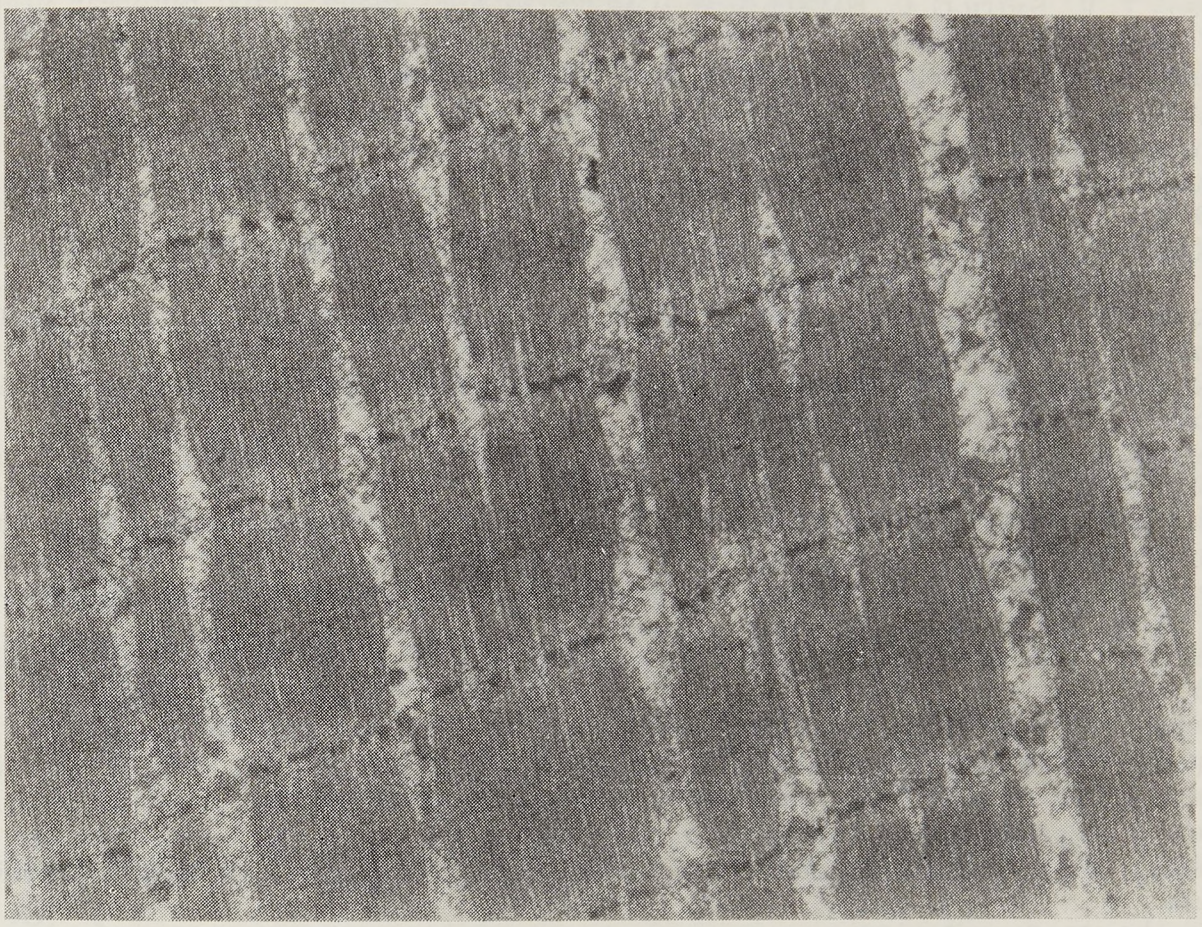

FIG. 2. — *Electron micrograph of control muscle from the unstimulated side of the same carcass as shown in Figure 1.*

1980). Results of these studies showed that the electrically stimulated sides had an initially higher temperature than non-stimulated sides and the electrically stimulated sides had a more rapid rate of chilling which produced the desired temperature in the electrically stimulated carcass up to two hours sooner.

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Research on low voltage electrical stimulation of beef carcasses in Sweden

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Fast chilling methods introduced

During the last 15-20 years the chilling speed of beef carcasses has been gradually increased in Sweden. The grading system favours the production of lean carcasses. The combination of these two facts has resulted in quite fast temperature fall in beef carcasses *post mortem*. In the middle of the seventies investigations showed that cold shortening conditions existed in several Swedish slaughterhouses in parts of the carcasses (fig. 1).

Reports from other countries (CHRYSTALL and HAGYARD, 1976; CARSE, 1973; SMITH *et al.*, 1977) suggested electrical stimulation as a possible means to

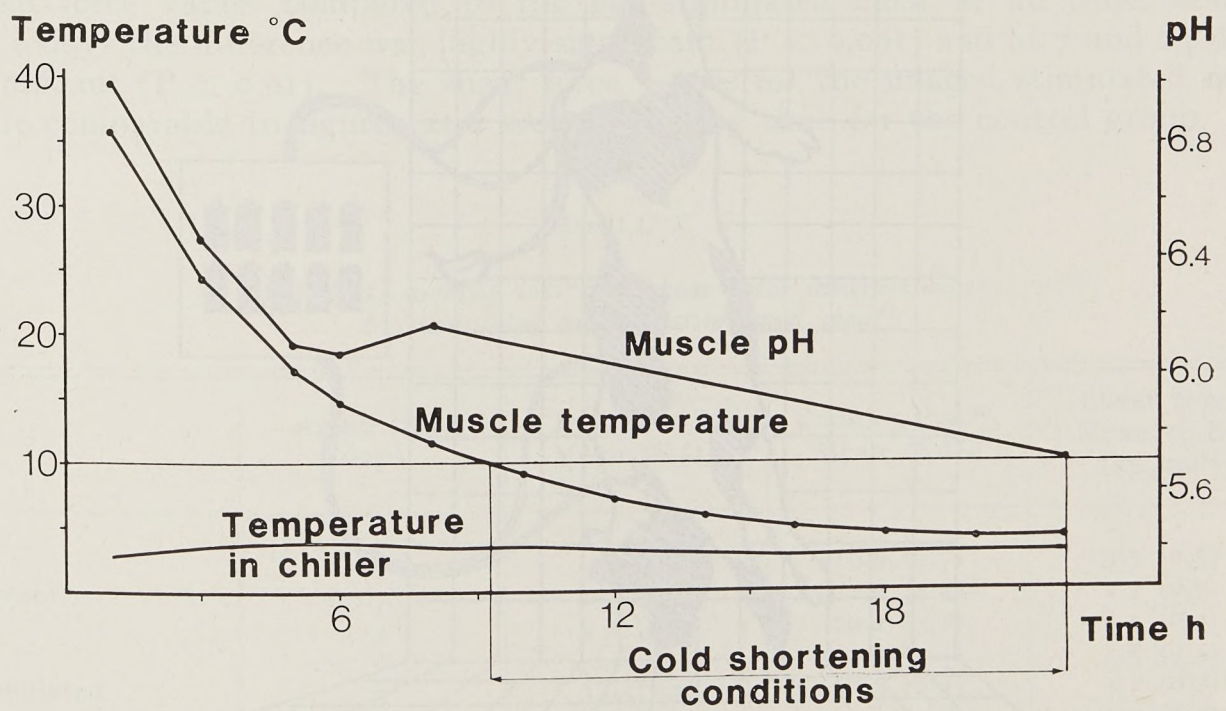

FIG. 1. — Post-mortem *temperature and pH in* M. longissimus dorsi *of beef carcasses under Swedish commercial conditions. Mean values from 21 carcasses.*

avoid cold shortening. However, it was considered undesirable, if not impossible, to introduce high voltage systems in Swedish slaughterhouses mainly for safety reasons. We therefore started to evaluate the possibility of using low voltage stimulation systems (voltages below 50 V RMS).

Low voltage stimulation showed promising results

The first experiments with low voltage stimulation were performed at the KBS abattoir in Kristianstad in 1977. A primitive stimulator was connected to the carcass via four needle electrodes (fig. 2) to avoid high and varying contact resistance (Jonsson *et al.*, 1977).

The results, exemplified in table 1, were encouraging and it was decided to further develop the method. This was to be carried out in cooperation between KBS, The Swedish Meat Research Institute and MIT AB, a Swedish company specialized in slaughterhouse equipment.

A thorough meat quality evaluation of low voltage stimulated beef was performed. Thirty lean young bulls with carcass weights ranging between 210 and 340 kg were used. Immediately after stunning and exsanguination twenty carcasses were electrically stimulated during 60 s with short bursts of high frequency peaks in pulses, 1 puls /s and a peak voltage of 80 V. Ten carcasses were used as

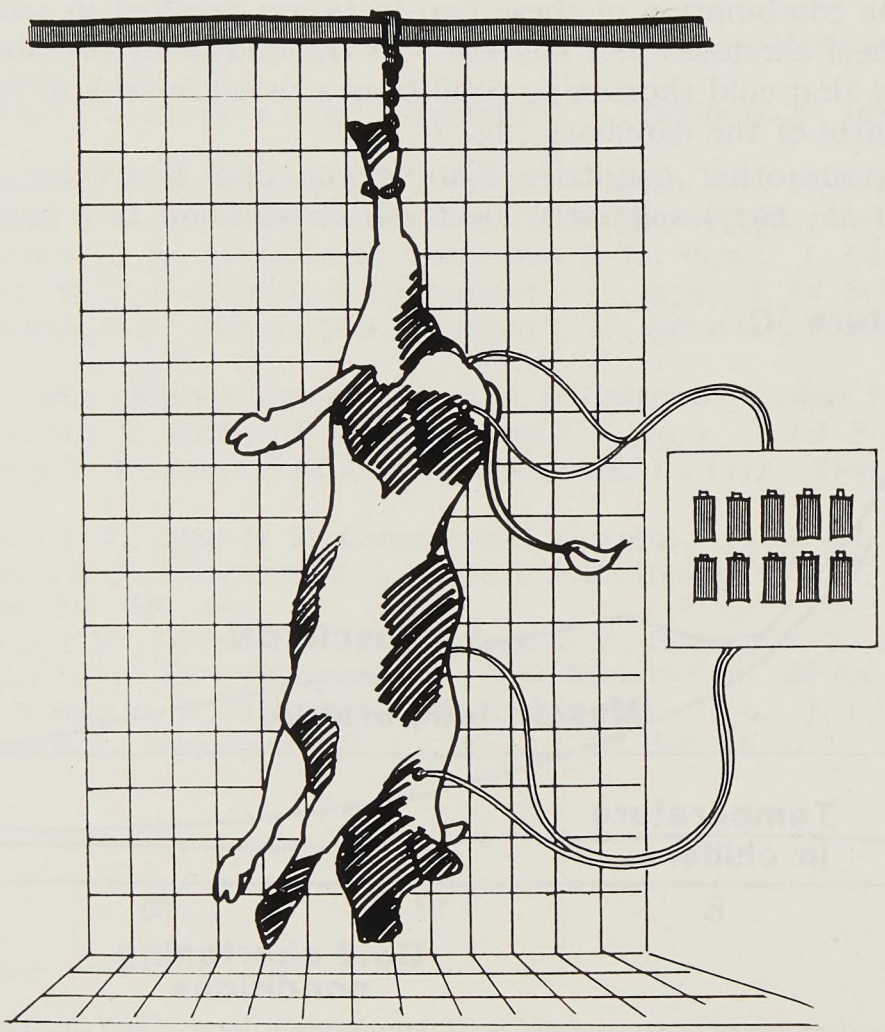

Fig. 2. — *Experimental set-up for electrical stimulation*

controls and not stimulated. After dressing and splitting, the carcasses were chilled at — 2 °C for 4 hours before transferring to + 2 °C for a further 24 hours. After chilling *M. longissimus dorsi* was removed, cut into steaks, vacuum packed and kept in + 2 °C for 14 days. Samples were removed and analysed immediately after cutting (0 days) and after 7 and 14 days of storage. Traits evaluated were drip and cooking losses, shear force, bacteriological standard and palatability.

TABLE 1

Shear force measurements in M. longissimus dorsi from conditioned stimulated and rapidly chilled carcasses (JONSSON *et al.*, 1977)

	Shear force (lb) Mean (S.D.)	
	Cutting day	14 days of aging
High temperature conditioning. . . .		
10 °C	6.09 (1.07)	3.37 (0.65)
14 °C	5.60 (0.85)	3.25 (0.61)
Low voltage electrical stimulation . .	6.17 (0.21)	2.58 (0.27)
Normal treatment.	5.78 (0.42)	3.82 (0.74)

Results of meat quality evaluation

Results from determinations of shear force, drip and cooking losses are summarized in table 2. The electrically stimulated meat showed considerably lower shear force values compared to the non-stimulated meat at all times tested. At 0 days the difference was highly significant ($P < 0{,}001$) and at 7 and 14 days significant ($P < 0{,}01$). The shear force values for the unaged stimulated meat were comparable to figures at 2 weeks of aging time for the control group.

TABLE 2

Drip loss, cooking loss and shear force measurements in stimulated and nonstimulated meat

	Aging time days	Drip loss % Mean (S.D.)	Cooking loss % Mean (S.D.)	Shear force Mean (S.D.) (kg/cm²)
Control.	0		19.0 (4.7)	3.5 (0.5)
	7	2.7 (1.3)	21.6 (3.3)	2.7 (0.7)
	14	3.2 (1.8)	19.2 (3.1)	2.4 (0.4)
Stimulated	0		22.0 (3.4)	2.5 (0.5)
	7	3.6 (1.2)	19.3 (2.4)	2.2 (0.2)
	14	4.9 (1.7)	20.1 (3.8)	1.9 (0.2)

Drip losses during vacuum storage were slightly higher for the electrically stimulated meat. The difference was almost significant ($P < 0,05$) only after aging for 14 days. Cooking losses were comparable for both treatments. Only small and insignificant differences in bacteriological counts could be detected.

Results of the palatability evaluations are shown in table 3. The palatability traits tested were in most respects in favour of electrical stimulation. Differences in tenderness were highly significant (0 and 14 days) or significant (7 days), electrically stimulated meat being the more tender. This was also the case for juiciness and flavour except after 14 days of aging when the difference was not significant ($P > 0,05$). Results from triangle test comparisons also indicated that there was a distinct difference in quality between the stimulated and not stimulated meat.

From these and other results we concluded that low voltage stimulation would indeed be beneficial in Swedish slaughterhouse practice. The main advantages were: 1) Improved and more uniform tenderness in marketed beef meat, 2) shorter aging time and less storage capacity needed, 3) fast chilling rates could be retained or even increased without risk for cold-shortening.

TABLE 3

Sensory evalution of stimulated and nonstimulated meat

	Aging time days	Tenderness Mean (S.D.)	Juiciness Mean (S.D.)	Flavour Mean (S.D.)
Control	0	2.15 [1] (0.89)	5.20 (0.69)	6.84 (1.24)
	7	4.32 (2.85)	5.56 (1.67)	7.09 (0.89)
	14	5.84 (3.09)	7.14 (1.31)	8.29 (0.70)
Stimulated	0	4.10 (1.42)	6.84 (1.00)	7.64 (0.72)
	7	7.00 (2.09)	6.62 (1.06)	8.56 (0.65)
	14	8.41 (1.15)	6.85 (1.31)	8.46 (0.73)

(1) Number on a 12-point hedonic scale where 0 is the worst and 12 the best quality.

Optimal conditions for low voltage stimulation

In the development of a commercial stimulation unit several experiments were carried out in order to find optimal stimulation conditions. Difficulties were encountered partly due to the fact that initially we were not aware of the exact nature of the pulses produced by the original primitive equipment. From our own experiments and the results of other workers (NILSSON *et al.*, 1979; RUDÉRUS 1980; SWATLAND, 1977) we finally came to the conclusion that successful low voltage stimulation had to be made as early as possible and that the pulses should be designed to stimulate the intact nervous system.

Good results were obtained by applying very sharp peaks at a frequency of about 14 Hz (RUDÉRUS, 1980). The pulse characteristics of the equipment now in use are shown in figure 3.

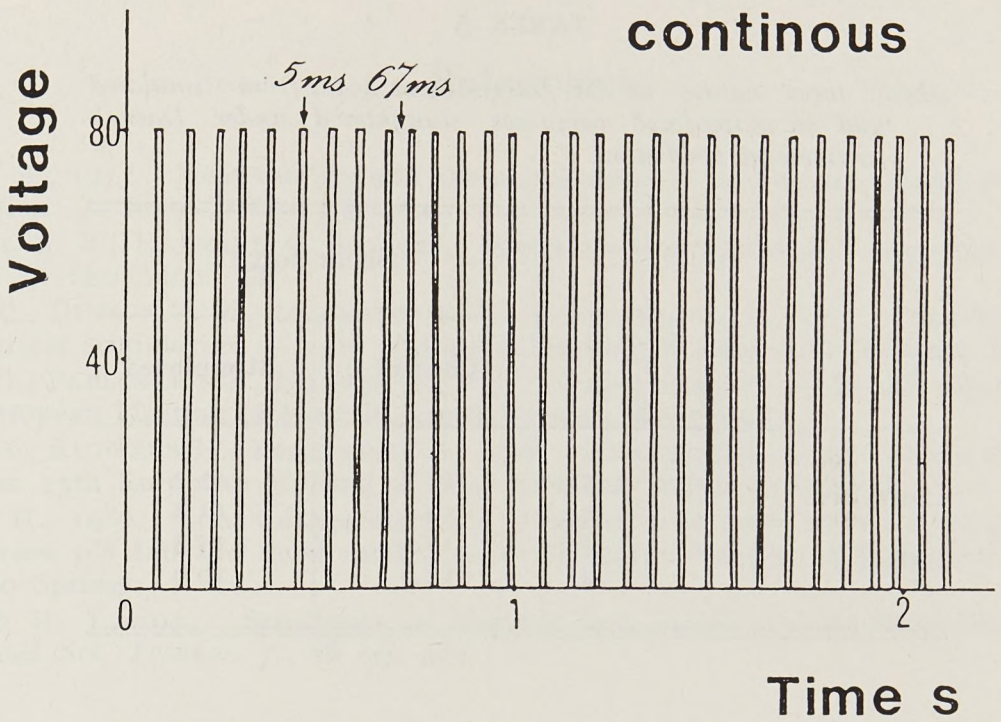

FIG. 3. — *Pulse characteristics of commercial equipment for electrical stimulation*

New results confirm earlier conclusions

Results confirming the earlier conclusions of the benefits of low voltage stimulation were recently obtained with a commercial unit in experiments in cooperation with the Danish Meat Research Institute (BUCHTER *et al.* unpublished results). Some of the results are shown in tables 4, 5 and 6. Fast pH fall was obtaine das before but there were slightly less differences in tenderness between stimulated and nonstimulated samples due to the slower cooling program used in commercial practice in Denmark. This indicates that local conditions are important when

TABLE 4

pH values in two muscles one hour after slaughter in stimulated and nonstimulated carcasses

Danish experiments	pH 1	
	Cows	Bulls
M. longissimus dorsi		
Control.	6.4	6.8
Stimulated	5.6	5.9
M. semimembranosus		
Control.	6.9	6.9
Stimulated	5.6	5.8

TABLE 5

Shear force values in M. longissimus dorsi *from stimulated and nonstimulated carcasses slaughtered under Danish commercial conditions*

		Shear force	
		Control	Stimulated
Cows	7	21.3	15.3
	14	17.1	12.3
Bulls	7	17.5	15.5
	14	12.8	10.9

TABLE 6

Sensory evaluation of the samples described in table 5

Scale : — 5 to + 5 where — 5 is the worst and + 5 the best quality.

	Danish experiments					
Aging time days	Tenderness		Juiciness		Flavour	
	Cows	Bulls	Cows	Bulls	Cows	Bulls
7 Control	— 1.8	0.10	1.2	2.7	1.6	1.6
7 Stimulated	— 0.3	0.01	1.6	2.6	1.6	2.0
14 Control	— 1.1	0.51	1.4	2.4	1.4	1.9
14 Stimulated	0.2	1.15	1.4	2.3	1.6	2.0

possible benefits of low voltage electrical stimulation are appraised. Several factors influence the over all effect of electrical stimulation and these sometimes differ considerably between plants.

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Several persons at Kristianstad Blekinge Slakteriförening (KBS) and at the Swedish Meat Research Institute have participated in the work described in this paper. We gratefully acknowledge their contributions.

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Characteristics and utilization of pre-rigor meat

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Hot boning of meat has been known for centuries but has gained worldwide interest in the last years for different reasons. The most recent interest is due to the need to save energy. In the last century the reasons for hot boning of beef were hygiene and a high quality of the products if this meat was processed immediately. In the southern part of Germany for instance it was a widespread custom in the first half of this century to cut the freshly slaughtered animal within the first 4-8 hours after death as the sausages made from this meat had an excellent water binding capacity.

As 50 p. 100 of the meat we eat in Germany is consumed as meat products we are mostly concerned with the problems of water retention and water binding of hot boned meat.

I will report here about our research on the biochemical changes in beef and about their influence on the water binding capacity. Afterwards I will report about the influence of temperature during the early post mortem period and ageing on water binding.

Biochemistry of the muscle after death

In a living animal the muscle receives energy-rich compounds like carbohydrates and O_2 via the blood stream. The energy-rich compounds are oxidized and ATP as a high-energy compound is synthetized. Metabolites and CO_2 are transported off by the blood. The pH of the living muscle stays around 7-7.4 and ATP is used for muscle contraction and a wide variety of metabolic processes. Some of the carbohydrates are stored as glycogen and some of the energy of ATP is stored as creatine phosphate in the muscle. At the moment of death the blood stream stops, and energy-rich compounds and oxygen are no longer provided by the blood. Also metabolites are accumulating in the muscle. Due to this the muscle uses up its resources of glycogen and CP to keep its metabolic processes in a state comparable to the living muscle. As it lacks oxygen the breakdown of glycogen switches from the aerobic to the anaerobic state producing now lactate and

protons instead of CO_2 and H_2O. As protons are produced the pH drops from pH around 7 at the moment of death to pH around 5.5 after using up all glycogen. pH 5.5 is in normal muscles the final pH. If there is a lack of glycogen the fall of pH is smaller and even sometimes nearly none. If this is the case we talk about dark cutting beef or DFD-meat. I will discuss here only the normal case where the pH drops to around 5.5 After the exhaustion of CP and glycogen also the resynthesis of ATP will end.

In figure 1 you see the course of several metabolites in the first 24 hours p.m. As the pH falls from pH 6.6 after 2 hours p.m. to 5.6 at 24 hours p.m., the amount of the lactate increases from 5 to about 60 µ Mol/g tissue. With this increase of lactate the glycogen concentration diminishes. The 4th parameter the R-value

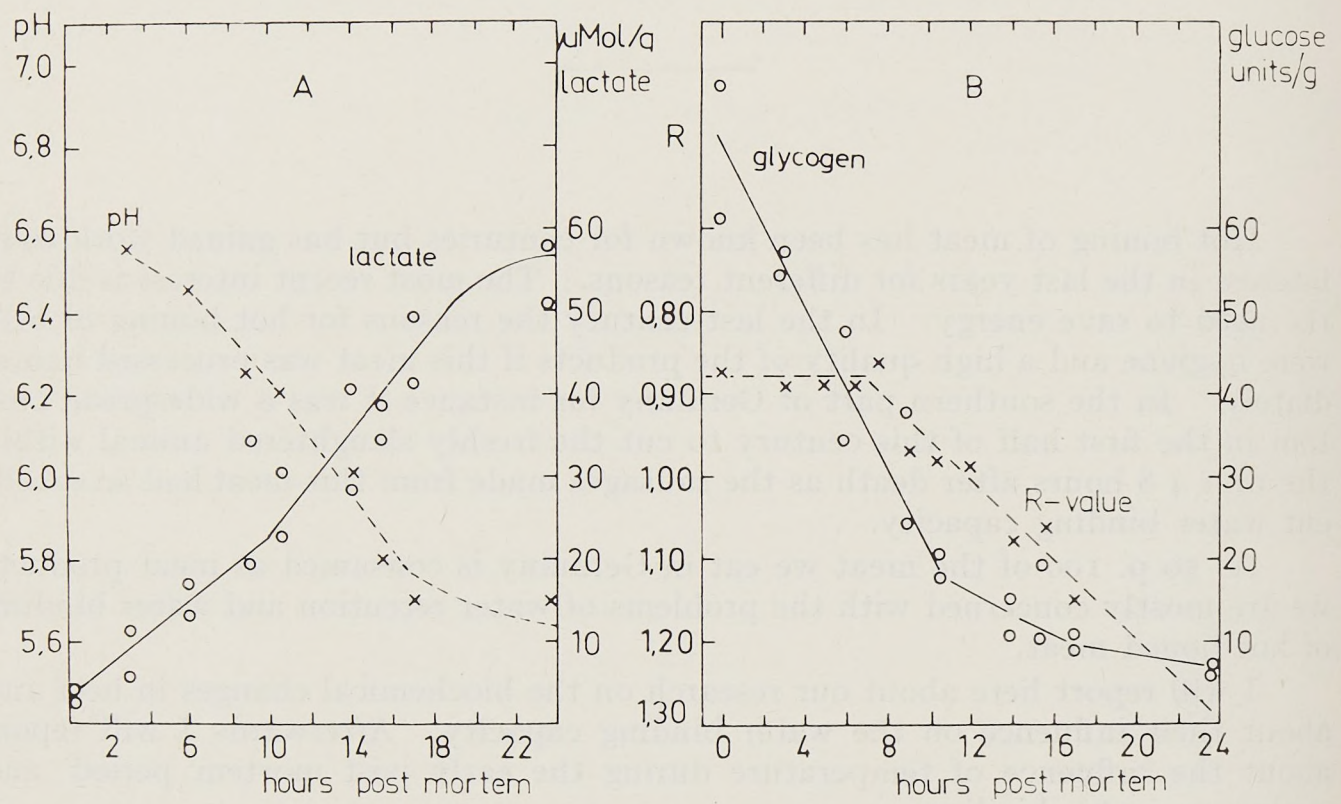

FIG. 1. — *Influence of time post mortem on the change on 4 biochemical compounds in bovine neck muscle (R is an indicator of ATP concentration in the muscle). Temperature 20 °C.*

is an easy measurable indicator of the ATP concentration (HONIKEL and FISCHER, 1977). Low R-value means high ATP concentration and vice versa. In the beginning the ATP concentration stays high and fairly constant, after 8 hours in this case it starts dropping. So at the moment of death we have a high pH, a high concentration of glycogen and a considerable amount of ATP. After 15-20 h we have a low pH, very little glycogen and little or no ATP. In the state when ATP and pH are high the muscle behaves physically or better physiologically very much different from the state when ATP and pH are low. This state we call prerigor or ante-rigor state, at low ATP and low pH we speak of the rigor or post rigor state. What influence have these changes on the behaviour and function of the muscle structure?

Biochemical processes and muscle structure

The basic unit of muscle fibril consists of the thick filaments, overlapping with the thin filaments and the Z-lines on both sides. These Z-lines are the limits of the basic units, the sarcomeres. The thick filaments consisting of myosin are between the thin filaments consisting of actin and the control proteins troponin and tropomyosin. They overlap with each other and slide into each other on contraction.

Contraction and extensibility of the muscle are only possible if ATP is present in the muscle fibers. After the exhaustion of ATP the thick and thin filaments combine to the actomyosin complex and the filaments cannot slide along

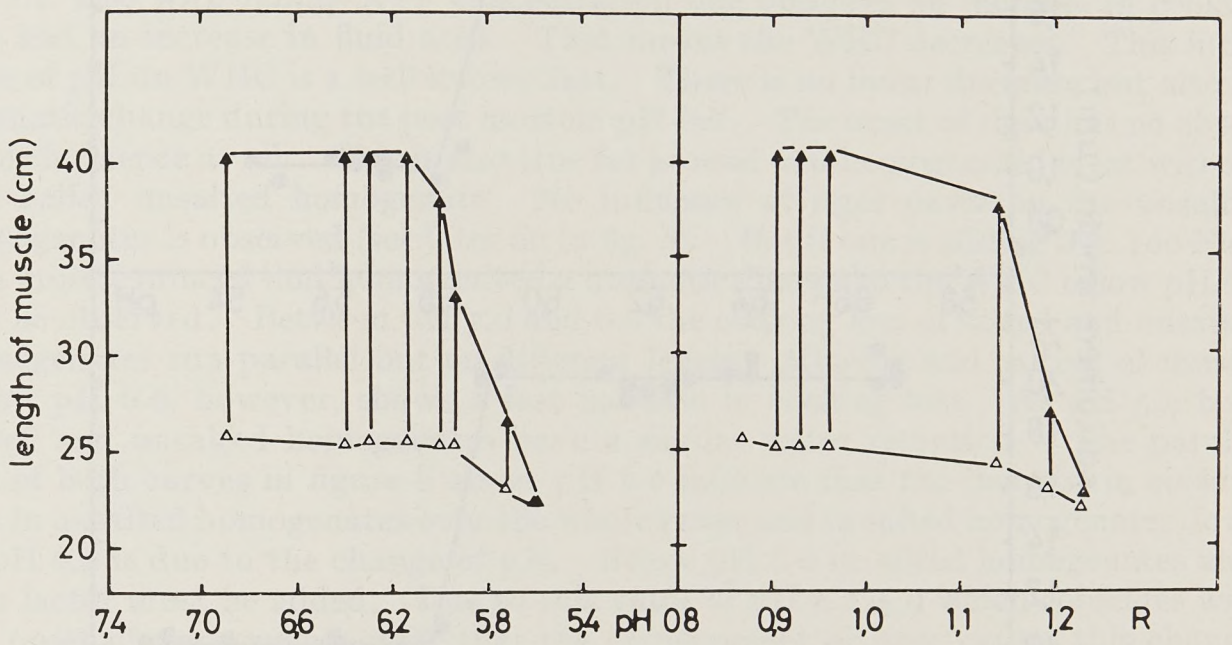

FIG. 2. — *Change of length in slaughterfresh bovine neck muscle without or with load (2 kg) in dependence of pH and R-value.*

(Δ) without load, (▲) under load.
Temperature 21 °C.

each other. The muscle gets stiff and looses its extensibility. This loss of extensibility can be measured easily with a simple technique.

An isolated muscle is removed from the carcass within 30-45 min. after death and hang up freely on one end. It has a certain length. On the other end we hang now a weight of about 2 kg and due to this weight the muscle stretches. After removal of the weight the muscle returns to its original length. If you measure the length of the muscle with and without load during the next few hours and also measure pH and R-value as an indicator for the ATP concentration you get the results shown in figure 2.

From pH down to pH 6.0-5.9 the length of the muscle without load stays at 25 cm and with load at 40 cm. Below pH 6.0 the muscle without load shortens to 22 cm and at the same time looses its extensibility under load. At pH 5.5 applying the weight does not lengthen the muscle anymore. The turning point is pH 6.0. Plotting the muscle length vs. the R-value shows that the turning poiht is at

R = 1.1 which is equivalent to an ATP concentration of about 1 μ Mol /g muscle. Loss of extensibility at the onset of rigor mortis occurs when the ATP concentration has dropped from 4-10 μ Mol ATP /g to about 1 μ Mol ATP /g. In normal muscle this ATP concentration is equivalent to pH 6.0 /5.9, which is easy to measure and which I will take furtheron as an indicator for the onset of rigor mortis. But not only the muscle under load gets shorter at the onset of rigor, also the unloaded muscle shortens with rigor onset. This is called rigor shortening as it starts immediately before rigor occurs.

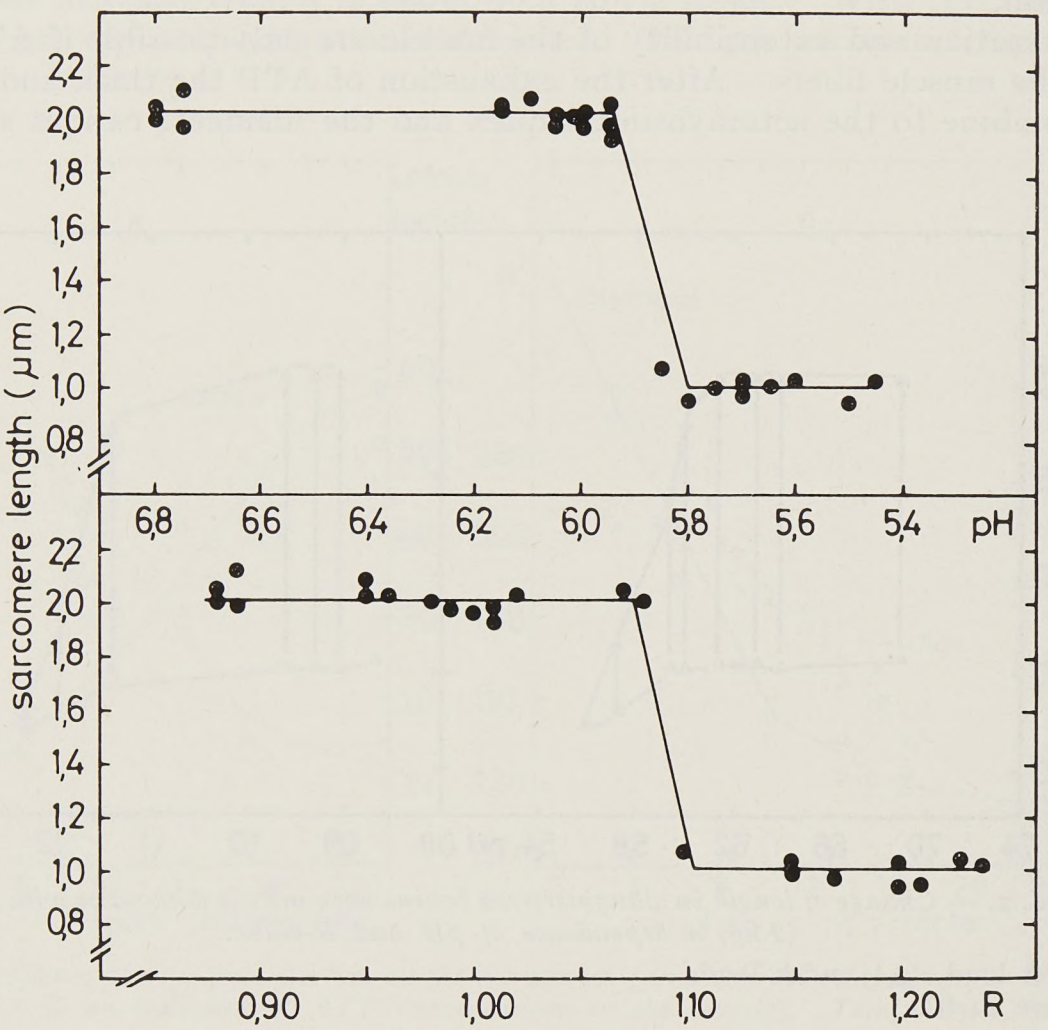

FIG. 3. — *Change in sarcomere length of bovine neck muscles during the changes post mortem. Temperature 30 °C.*

In figure 3 you see this rigor shortening even better. Here we measured the sarcomere length with a laser technique (VOYLE, 1971) of an unloaded muscle in dependence of its pH and R-value. At pH 5.9 and R-value 1.05 the sarcomere length decreases from about 2 μm to about 1 μm very suddenly. No change before and no change after reaching pH 5.9 or ATP concentration of about 1 μ Mol /g can be observed.

In summary: The rigor mortis sets in at an ATP concentration of 1 μ Mol ATP /g and at about pH 5.9. In these experiments at room temperature no shortening occurs above these values. It will be a different story au lower temperatures which I will report lateron.

Onset of rigor and water-holding capacity of beef

Finally we come to the topic of paper: To the question what influence have the biochemical and structural changes on the water-holding capacity (WHC) of meat? Let's look at first into meat as intact muscle. WHC can be measured in a lot of different ways. There are nearly as many methods as meat scientists work on this problem. We used several methods. One is the application of pressure in the filter paper press method of GRAU-HAMM (1957), the other is the cooking loss. With the press method you take 0.3 g of muscle put it on a filter paper, press it between two perspex plates for 5 min. and take the fluid area around the meat film as a measure of WHC. With the cooking loss you measure the weight loss of meat on cooking for 20 min. in a boiling water bath. With falling pH i.e. also with falling ATP concentration one observes an increase in cooking loss and an increase in fluid area. That means the WHC decreases. This influence of pH on WHC is a well known fact. There is no linear decrease but also no dramatic change during the post mortem pH fall. The onset of rigor has no observable influence at all. This is also true for ground and homogenized meat without salt called unsalted homogenate. No influence of rigor onset on the unsalted homogenates is observed (see later on in fig. 8). But if one is adding 2 p. 100 NaCl to a muscle minced and homogenized a dramatic change in the WHC below pH 6.0 can be observed. Between pH 7.0 and 6.0 the cooking loss of salted and unsalted homogenates run parallel but on different levels. Mincing and salting of muscle below pH 6.0, however, shows a fast increase in cooking loss. At pH 5.4 both salted and unsalted homogenates have a similar water retention. The parallel run of both curves in figure 8 above pH 6.0 indicate that the increase in cooking loss in unsalted homogenates over the whole range and in salted homogenates down to pH 6.0 is due to the change of pH. Below pH 6.0 in salted homogenates another factor must be added. Due to this value of pH 6.0/5.9 which coincides with the onset of rigor we conclude that the development of rigor causes this change.

Also at R-values below 1.05 equivalent to an ATP concentration of about 1 μ Mol ATP /g the cooking loss in salted homogenates is low. With the onset of rigor the cooking loss increases sharply. From this results we also conclude that the ATP concentration by itself has no immediate or direct influence on the WHC in salted homogenates. It seems to be an indirect influence. As long as the ATP concentration is high and keeps the thick and thin filaments apart the WHC is dependent on the pH only. After the depletion of ATP and the onset of rigor and the formation of the actomyosin complex the WHC of salted homogenates drops. Or expressed differently: ATP in the muscle prevents the rigor and keeps the WHC high.

You certainly ask, why is there an influence of rigor on the WHC of salted meat but no influence of rigor in the unsalted homogenate? Let me try to give you an explanation with figure 4.

Here you see in a scheme the system of thick and thin filaments in unsalted and salted homogenates at different pH values. In this scheme the distance between thick and thin filaments shall indicate a swelling of the myofibrillar structure resulting in a better water binding. At pH 7.0 in the prerigor state in salted and unsalted homogenates the actin and myosin filaments are fairly independent of each other. Without salt, however, they show only a relative limited swelling. With falling pH due to change in protein charges the distance between fibrils and

filaments gets smaller. Swelling and WHC is reduced. The formation of the actomyosin complex with the onset of rigor will have no dramatic effect, as the filaments are before rigor even near together. Salting, however, in the prerigor state lets the myofibrillar structure swell and disolve some of the protein. There is some decrease in swelling with falling pH. But as long as there is no AM-complex considerable swelling is possible. After rigor onset, however, no swelling can occur anymore within the rigid structure of the AM-complex.

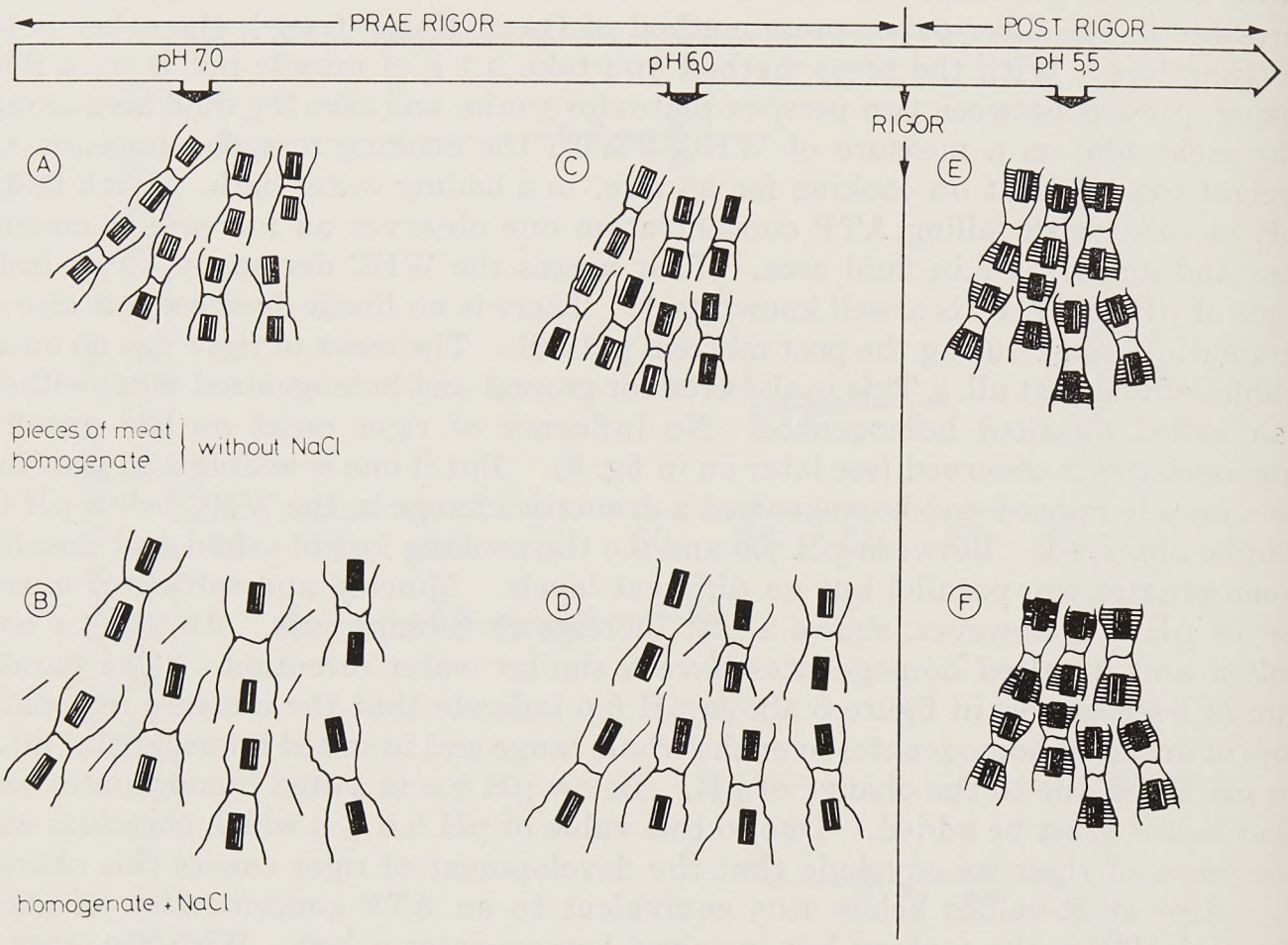

FIG. 4. — *Scheme of the influence of post mortem pH fall and salt in muscle homogenates. Swelling is indicated by a wider distance. At pH 5.5 crosslinking is indicated by cross-striation.*

Conclusions

Salting of slaughterfresh bovine muscle before the onset of rigor causes an extreme amount of swelling in the muscle structure and the WHC of meat products made there off is high. In muscles of normal condition (not dark cutting beef) the moment of rigor onset is at about pH 6.0 /5.9 which is in practise easy to measure compared to the actual ATP concentration which, however, would give a more direct information about the rigor onset.

pH 6.0 /5.9 is the true biochemical turning point for the change on WHC. We in the lab can work very fast with small amounts of meat. In meat plants, however, one works with tons of meat and one needs more time for grinding and salting. So if one intends to use slaughterfresh meat for sausage making take a higher pH value as the lowest limit. pH 6.4, 6.3 or 6.2 may be the limit it depends how fast one is able to grind, salt and homogenize the meat.

Influence of temperature on the water-holding capacity of meat

What influence has the temperature on the water binding of meat? All experiments (except the ones in fig. 3) reported so far have been carried out at room temperature around 20 °C. But in practise after death carcasses are not stored at 20 °C. In the first hours after death the muscle is at higher temperatures of 30 °C or even above. At the onset of rigor after 8-12 hours temperatures below 15 °C are observed especially if the carcasses are cooled effectively. We have therefore expanded our research to the whole temperature range between 0 and 30 °C and also extended our measurements for up to 7 days in order to show the influence of temperature for a longer period of time. Cooling of carcasses is necessary for hygienic and desirable for economic reasons as the faster the temperature is lowered the smaller is the weight loss as less water evaporates. Extreme cooling, however, has an quality disadvantage as the muscle shortens which results in toughness. This phenomena is called cold shortening and is prevented as you know by electrical stimulation. I want to recall the biochemical facts that cold shortening occurs only in muscles before the onset of rigor and below 10 °C in the muscle. Especially the so called red muscles are prone to shortening but also less red muscles of pork can exhibit cold shortening but at lower temperatures below about + 4 °C (Fischer *et al.*, 1980).

On cold shortening the overlapping of the filaments increases on shortening. At the onset of rigor in such a cold shortened muscle the stiffness of these muscles increases due to a higher degree of crosslinking. Above you have heard that the crosslinking has a decisive influence on the WHC of salted homogenates but not on intact muscle. So one could expect that the degree of crosslinking has an influence on WHC of salted homogenates.

Temperature: Its influence on biochemical processes and shortening

Everyone knows lowering the temperature decreases the velocity of chemical and biochemical reactions. Therefore cooling of carcasses should slow down the velocity of the pH fall in a carcass. In a beef muscle this is true only for temperatures above 6-8 °C. Below this temperature within the first 3-4 hours p.m., however, an acceleration is observed. In the same time and temperature range cold shortening occurs. Probably due to the contraction glycolytic processes are accelerated and the production of lactic acid increases as more ATP is used for the contraction. But the interesting thing is that this is true on lyin the first 4-6 hours p.m. (fig. 5).

If one takes an isolated slaughter fresh muscle, cools it rapidly to 0.5 °C and measures its pH, one observes a rapid pH fall. But after 4-6 hours the pH fall slows down. At 7,5 °C and 14 °C you observe a lag phase for 2-4 hours and then an increase in pH fall, passing the pH fall at 0.5 °C at about 12 hours p.m. Even at 30 °C the pH fall is slower in the beginning than at 0.5 °C. However, if you look at the time when the rigor onset occurs you see that rigor sets in at first at

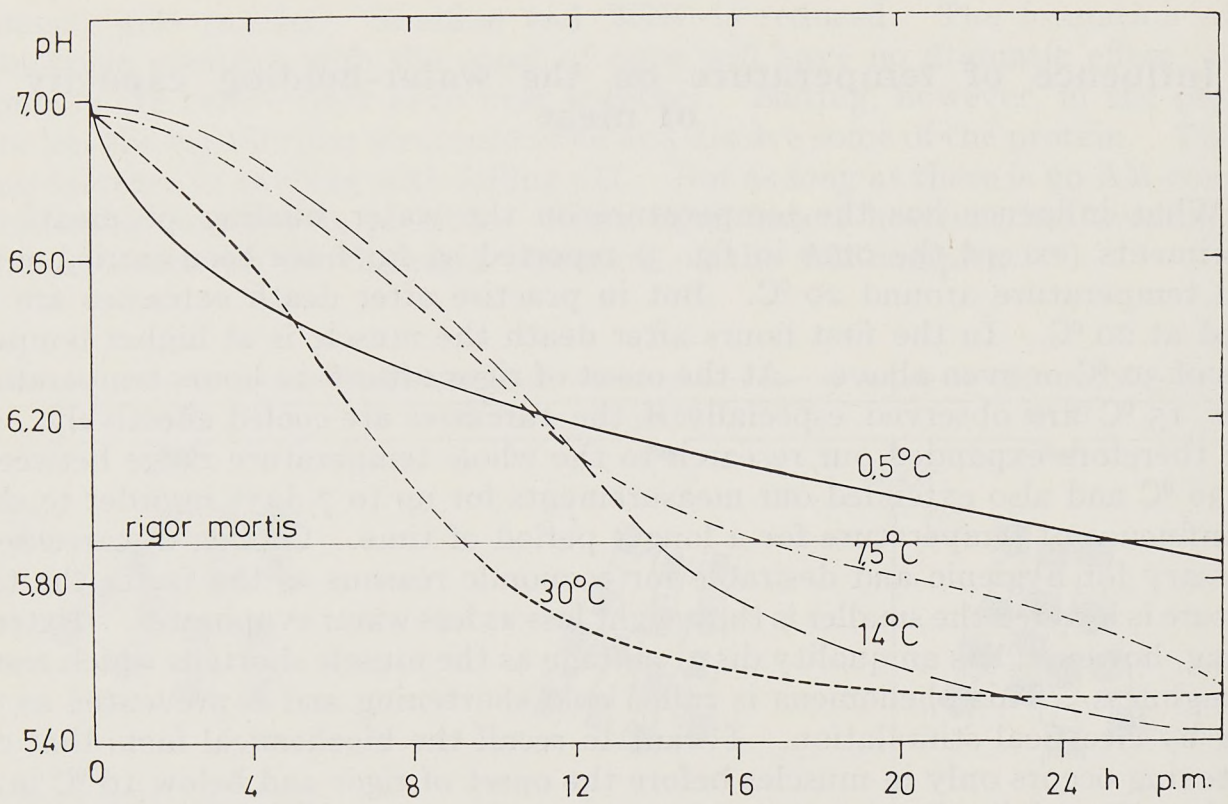

FIG. 5 — *pH-time curve of bovine neck muscles incubated at different temperatures.*

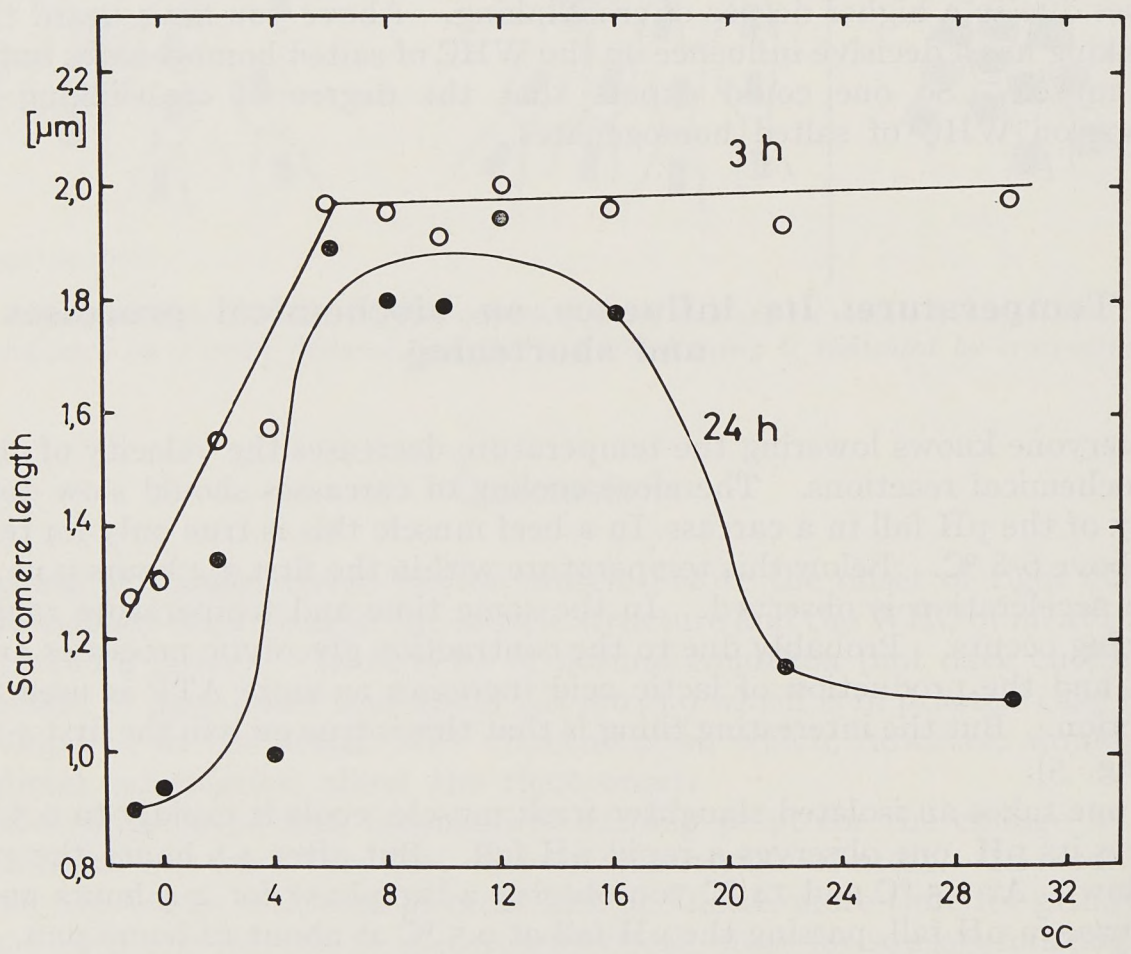

FIG. 6. — *Sarcomere length of bovine neck muscles at 3 and 24 hours post mortem at different temperatures.*

30 °C, then at 14°, 7,5° and finally at 0.5 °C. So the time of onset of rigor is temperature dependent as one would expect without knowledge of cold shortening.

If you measure the sarcomere length of bovine muscles at different incubation temperatures you see in figure 6 that below 6-8 °C the muscle shortens within the first 3 hours to values of 1,25 µm at — 1 °C. Above 8 °C the sarcomere length stays at about 2 µm up to 30 °C. After 24 hours when the final pH is reached and rigor has occured, the muscles have shortened in any case. But below 8 °C and above 15 °C muscle has shortened considerably. Below 8 °C it is mainly due to cold shortening, above 15 °C the rigor shortening has occured.

Water-holding capacity and shortening of unsalted muscle

I have reported above that at 20 °C intact muscle and homogenate without salt lowers the WHC with falling pH without influence of rigor onset. What influence have higher and lower temperature on this behaviour? Lowering the temperature lowers the velocity of pH fall. But if you compare the cooking loss of intact muscle dependent of pH regardless of the time p.m. you get an interesting result shown in table 1.

TABLE 1

WHC (cooking loss in p. 100) of muscle, unsalted and salted muscle homogenate at different pH and temperatures

Temp. °C	pH 6,8			pH 6,1			pH 5,9			pH 5,5		
	Muscle	Homogénate		Muscle	Homogenate		Muscle	Homogenate		Muscle	Homogenate	
		Unsalted	Salted		Unsalted	Salted		Unsalted	Salted		Unsalted	Salted
0,5	34	52	24	42	58	36	44	59	40	—	—	55
4	34	50	16	40	53	31	41	55	34	44	60	51
5	36	53	20	41	56	32	42	56	37	45	57	50
7,5	37	51	28	40	56	29	42	57	35	45	60	49
10	27	49	22	38	55	28	40	55	35	45	55	51
14	33	53	22	39	54	22	41	55	22	44	58	53
17	32	49	21	40	57	36	43	57	41	44	58	54
20	37	48	27	42	58	36	43	58	40	44	60	51
20	35	42	16	39	50	24	41	42	27	43	57	48
23	33	55	31	40	57	32	42	59	33	44	59	54
24	37	47	26	44	58	33	46	59	34	53	59	50
27	37	53	28	39	57	32	40	58	34	42	60	47
30	35	52	26	41	56	32	42	57	34	45	60	56
$\bar{X}$	34,4	50,3	23,6	40,4	55,8	31,0	42,1	56,7	34,3	44,8	58,6	51,5
S.D.	± 2,8	± 3,4	± 4,6	± 1,6	± 2,3	± 4,3	± 1,7	± 2,0	± 5,2	± 2,7	± 1,6	± 2,7

At pH 6.8 and 6.1, that means prerigor, you see that at temperatures where cold shortening occurs, the cooking loss is the same as above 10 °C.

Also onset of rigor at pH 5.9 and even at the moment of reaching the final pH 5.5 no influence of temperature can be observed.

Neither cold shortening nor rigor shortening at pH 5.9 have an influence despite a different degree of shortening and crosslinking.

The conclusion is that in muscle during the post mortem pH fall no influence on WHC can be observed despite a considerable change in muscle structure. pH only influences its WHC.

Now meat is not processed and consumed immediately after reaching its final pH. It is aged for days or even weeks. Is there an influence of the temperature during this time of aging, where no or only little change of pH is observed? To study this we cut cubes of meat of about 30 g, put it in plastic pouches and

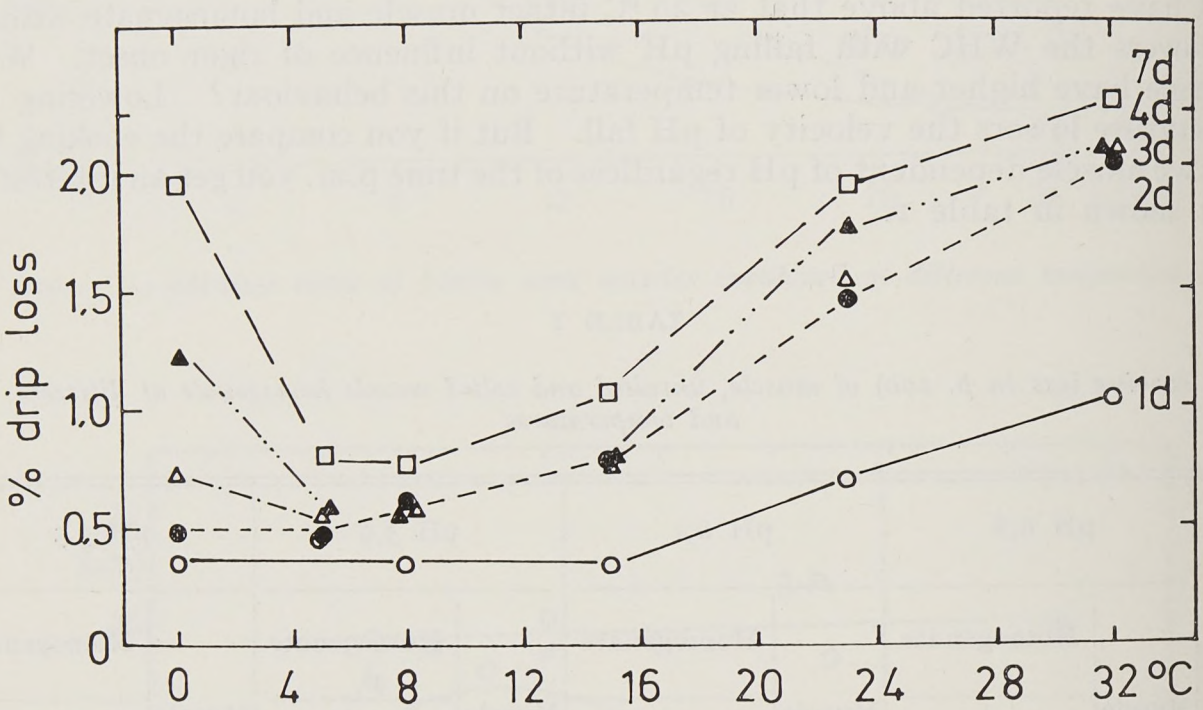

FIG. 7. — *Drip loss of bovine neck muscles incubated at the first day at 0° to 30 °C. After 24 hours the muscles were incubated up to 7 days at 4 °C.*

incubated it hanging freely at different temperatures up to 7 days and measured the drip loss during that time.

After day one there was as expected no influence of temperature. After 2 days the drip was higher at 23 and 30 °C. After 3,4 days the drip was higher at 0, 4, 15 and 22 °C with a minimum at 8-12 °C. These curves remind very much on the curves of sarcomere length as shown before where we observed the minimal shortening at 8-15 °C. Apparently shortening causes an increase in drip loss but only after several days. In order to prove this we did further experiments where we stored the meat at the first day at these different temperatures and put it after 24 hours at 4 °C, which is more like what happens in practise.

In figure 7 you observe a similar change in drip loss. Shortening which occurs at the first day increases the drip loss, even if all muscles are stored at the same temperature after 24 hours. The conclusion for the handling of beef is that meat which ages shows the lowest weight loss if the shortening of muscle during the first day is minimal. Muscle shortening increases drip loss but with a lag phase.

Water-holding capacity of salted meat

Contrary to intact muscle and unsalted homogenates, homogenates with salt exhibit an influence of rigor or crosslinking. Is there also an influence of the degree of shortening or crosslinking on WHC of salted homogenates? To study this we prepared salted homogenates of muscles incubated between 0 and 30 °C. In figure 8 and table 1 one can see that salting of homogenates exhibits during the

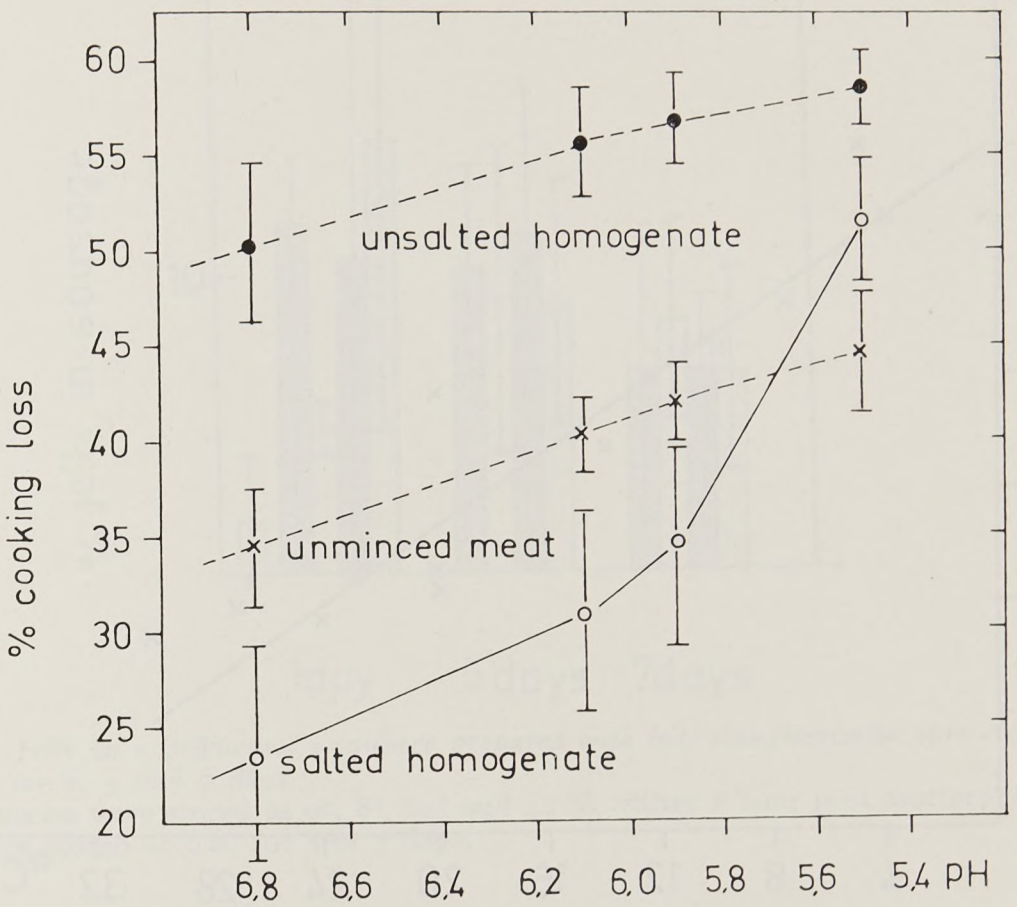

FIG. 8. — *Percent cooking in unminced meat (intact muscle), unsalted homogenate (50 p. 100 water added to the homogenized meat) and salted homogenate (50 p. 100, 6 p. 100 NaCl solution added) in dependence of pH at temperatures between 0 and 30 °C. Bars indicate the standard deviation. The figure is the graphic presentation of table 1.*

post mortem pH fall no significant influence of temperature. Again only rigor onset causes an increase in cooking loss.

The bars in figure 8 indicate the s.d. Intact muscle, unsalted and salted homogenates show between pH 6.8 and 6.1 the same pH dependence. At the onset of rigor the cooking loss of salted homogenates decreases rapidly. You see only pH and rigor have an influence on WHC of salted homogenates, there is no influence of shortening. So decreasing the temperature in slaughter fresh muscle and shortening has during the post mortem pH fall no influence on WHC.

But the velocity of pH fall and the time when rigor occurs is temperature dependent. So if in meat processing you intend to use hot boned meat immediately for sausage production rapid cooling or shock cooling extends the time where you

can use this meat before rigor onset. Figure 9 shows that the time necessary to reach pH 5.9 the moment of rigor onset is 4-6 hours at 30 °C but about 24 hours at 0 °C.

So rapid cooling is bad for aging of fresh meat, but it is good for the WHC in sausages.

This statement is valid for the immediate use of slaughter fresh meat for sausages but how about the influence of temperature or shortening on the WHC of salted homogenates after several days of storage of the meat as intact muscle.

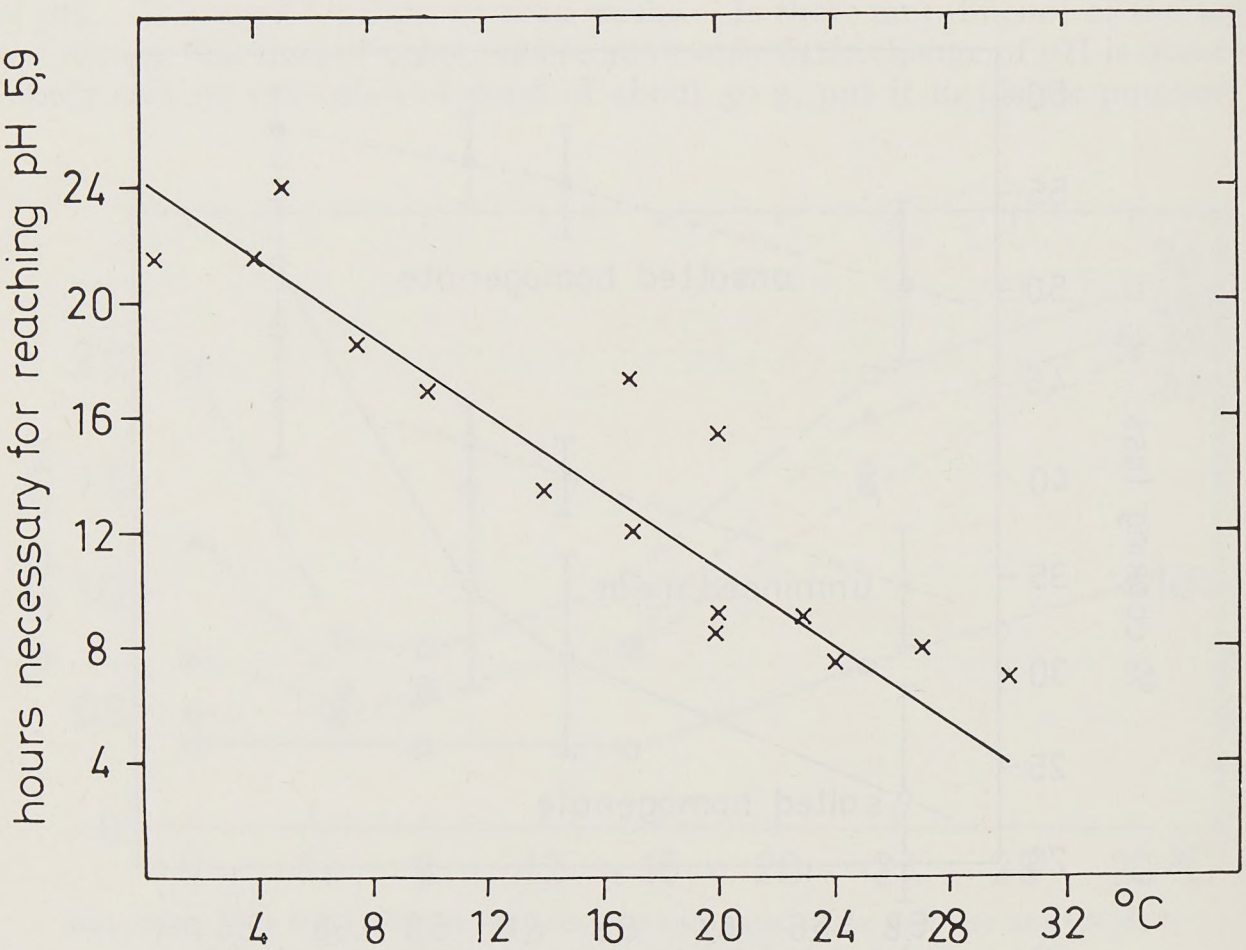

FIG. 9. — *Relation between muscle temperature and time to reach pH 5.9 (rigor onset) in bovine neck muscles.*

So we took meat, stored it at the first day at 0°, 5°, 12° and 22 °C and aged it after 24 hours up to 7 days at 0 °C and prepared salted homogenates of this meat The WHC of the salted homogenates shows no influence of shortening. You shorten the muscle, you get quite an amount of the drip, but the WHC of the salted homogenates made there off even by addition of the exudated drip shows no effect on the WHC. Now, you may argue that salted homogenates are no sausages which contain fat. So we made also sausages of this meat shown in fig. 10 and found no significant influence of shortening and storage in the sausages.

The amount of jelly in the sausages is after 5 and 7 days storage of the meat nearly the same for all 4 investigated temperatures.

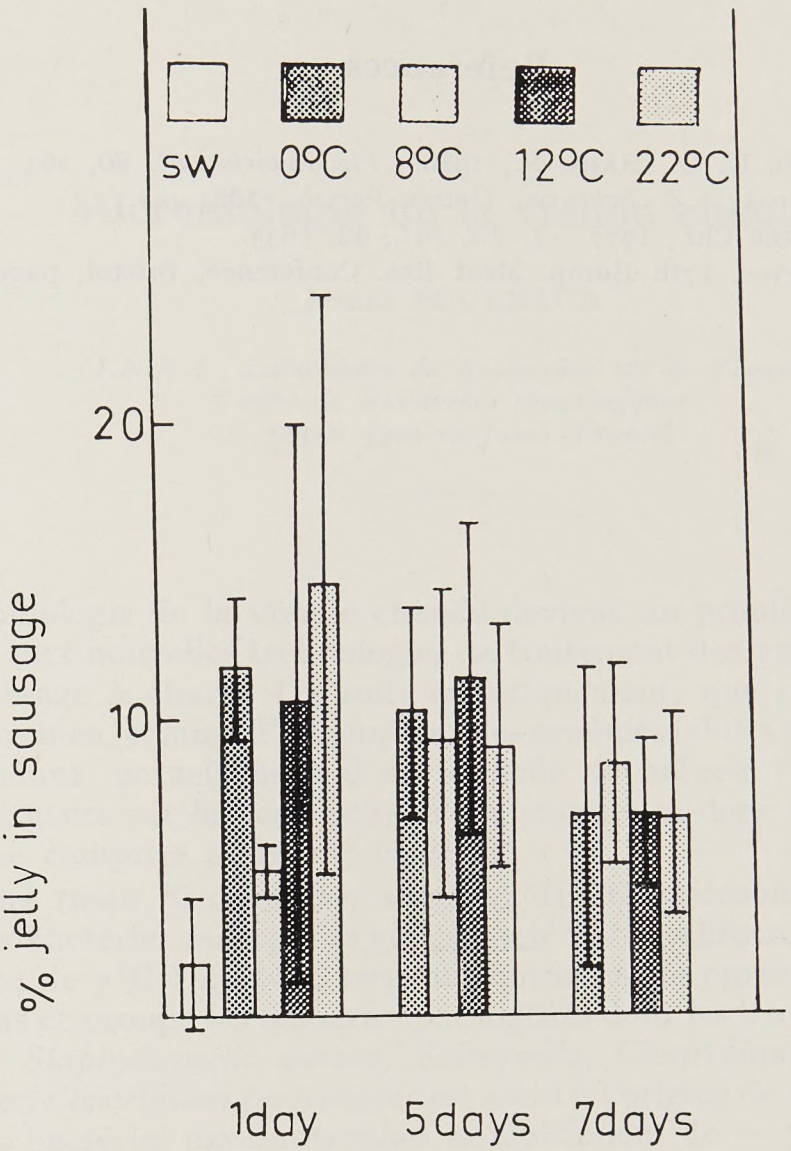

FIG. 10. — *Jelly in « Brühwurst » sausage prepared with beef slaughterwarm (pre rigor) or after storage for 1, 5 and 7 days.*
The muscles were stored at 0°, 8°, 12° and 22 °C within 1 hour post mortem for 24 hours and then stored at 0 °C for 5 or 7 days.

Conclusion

Shortening and rigor or crosslinking have a different influence on the quality of meat and meat products. If you are interested in tenderness of meat then shortening besides rigor increases thoughness. You should avoid excessive shortening either by moderate cooling or electrical stimulation.

The same is true for the WHC measured as weight loss of meat by drip. No immediate influence, but an influence after several days, cool slowly to 10-15 °C within the first 12-24 hours. If you intend, however, to use your meat for sausage production where WHC is most important there is no influence of shortening, here the onset of rigor is the decisive factor. Rapid cooling is not a disadvantage, in the contrary it allows you to extend the time of handling the meat for hot boning and salting in order to take advantage of the excellent WHC of slaughterwarm beef for sausage production.

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Microbiologie de la viande chaude

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La microbiologie de la viande chaude devient un problème de plus en plus important dû aux nouvelles technologies de traitement des viandes, en particulier celle du désossage à chaud. On sait, empiriquement, que la viande chaude se conserve moins bien, comme d'ailleurs tous les produits labiles placés à température élevée. Cependant, actuellement il est difficile de prévoir l'effet d'un écart de 1 °C de température sur la bactériologie des viandes et donc sur sa conservation dans une zone comprise entre 10° et 40 °C.

Cette zone 10-40 °C concerne, au point de vue microbiologie, 2 groupes de bactéries : les bactéries mésophiles et les bactéries psychrotrophes. Les premières se développent de 7 °C à 45-50 °C avec un optimum de croissance vers 37 °C. On rencontre dans ce groupe les bactéries pathogènes dont les bactéries d'intoxication alimentaire : *Staphylococcus aureus, Salmonella, Clostridium.* Parmi ce dernier genre bactérien *Clostridium perfringens* est aussi à l'origine de la putréfaction verte profonde. Les bactéries psychrotrophes se multiplient de — 5 °C à environ 37 °C avec un optimum situé entre 20 et 25 °C. Appartiennent à ce groupe des bactéries susceptibles de poisser les viandes, de les putréfier et de changer les pigments de rouge en vert. Toutes les bactéries ne se développent pas de la même manière dans leur plage de température. En effet, une bactérie suit un certain nombre de règles. Elle observe d'abord une phase de latence qui est une période d'adaptation au milieu; son nombre ne varie pas ou même parfois tend à diminuer. Ensuite elle se multiplie en se divisant par deux, chacune des cellules fille obtenue se divisant elle-même par deux. La phase de latence se trouve être plus ou moins longue, la croissance plus ou moins rapide selon divers paramètres tels que la température, la présence ou l'absence d'air (potentiel redox), l'acidité du milieu (pH). Quand tous ces facteurs varient comme c'est le cas d'une viande chaude en cours de refroidissement, le bactériologiste doit les étudier un par un puis ensuite les intégrer. Le meilleur exemple que l'on puisse donner est celui de *Clostridium perfringens* tel qu'il résulte des travaux d'INGRAM.

Le premier paramètre étudié est celui de la température (fig. 1). A 39 °C le doublement du nombre de bactéries se répète 4,5 fois par heure c'est-à-dire qu'en moins d'un quart d'heure le taux de contamination est multiplié par 2. A 37 °C le doublement a lieu 3,7 fois par heure, c'est-à-dire qu'en une heure la population croît d'un peu plus d'une puissance de 10. Le développement cesse pratiquement en-dessous de 20 °C.

Si on prend en compte la variation de température due au refroidissement de la carcasse, il est possible d'obtenir, en fonction du temps *post-mortem*, la vitesse

de multiplication (fig. 2) et donc de calculer le nombre de doublement en fonction de la rapidité de refroidissement de la carcasse. Ainsi, si l'animal est placé en chambre froide à 5 °C on obtient 33 doublements; s'il est placé à 15 °C, 67 doublements sont possibles dont 30 dans les 7 premières heures *post-mortem*. Trente doublements correspondent à une multiplication du nombre de bactéries de un

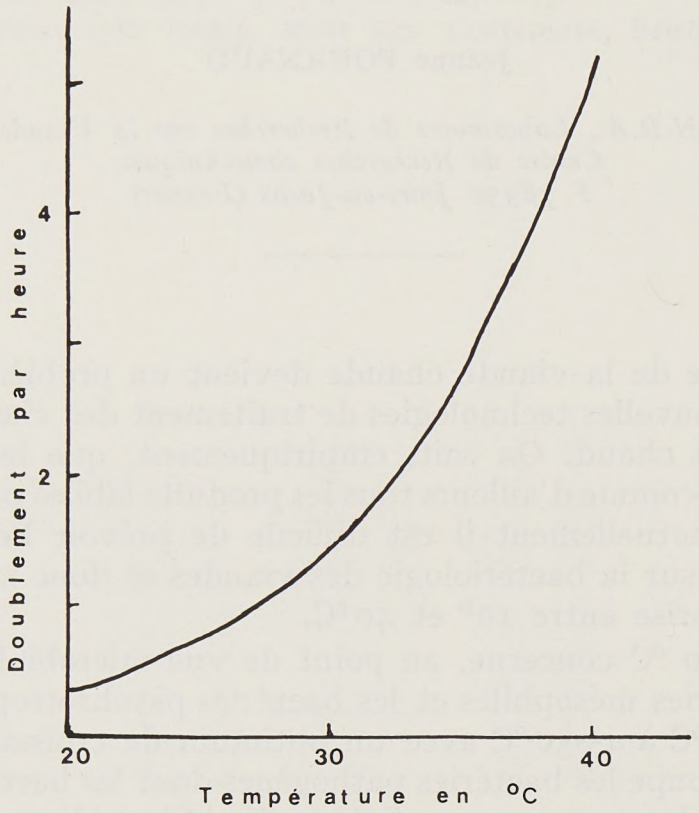

FIG. 1. — *Croissance de* Clostridium perfringens *à différentes temperatures*

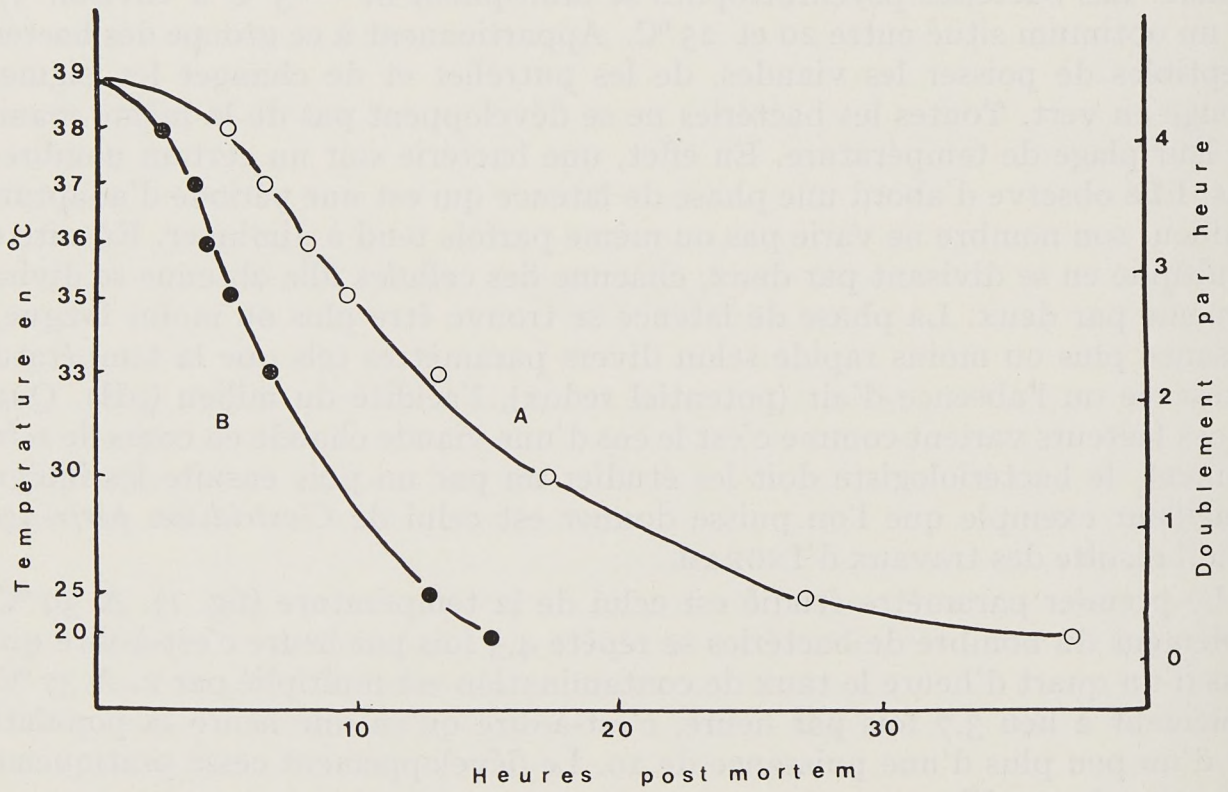

FIG. 2. — *Vitesse de croissance (doublement par heure) de* Clostridium perfringens *en fonction de la température de la carcasse et du temps* post-mortem. D'après INGRAM (1972)
A) Ressuyage à 15 °C. B) Ressuyage à 5 °C.

milliard (10^9). Si on admet la présence dans le muscle d'une cellule bactérienne pour 100 g le taux de pollution final serait de 10^8 bactéries par gramme donc présence de pourriture verte. Ceci n'arrive pas dans la pratique, il y a donc d'autres facteurs qui entrent en jeu.

Le premier apparaît être le potentiel d'oxydo-réduction. *Clostridium perfringens* appartient au groupe des bactéries anaérobies strictes. La viande, au moment de l'abattage, renferme de l'oxygène et son Eh est de l'ordre de + 230 mV. Pour ce potentiel, les essais de laboratoire montrent qu'il n'y a pas de croissance (fig. 3). Quand Eh décroît la phase de latence diminue; mais il faut attendre — 50 mV pour obtenir la multiplication sans période d'attente.

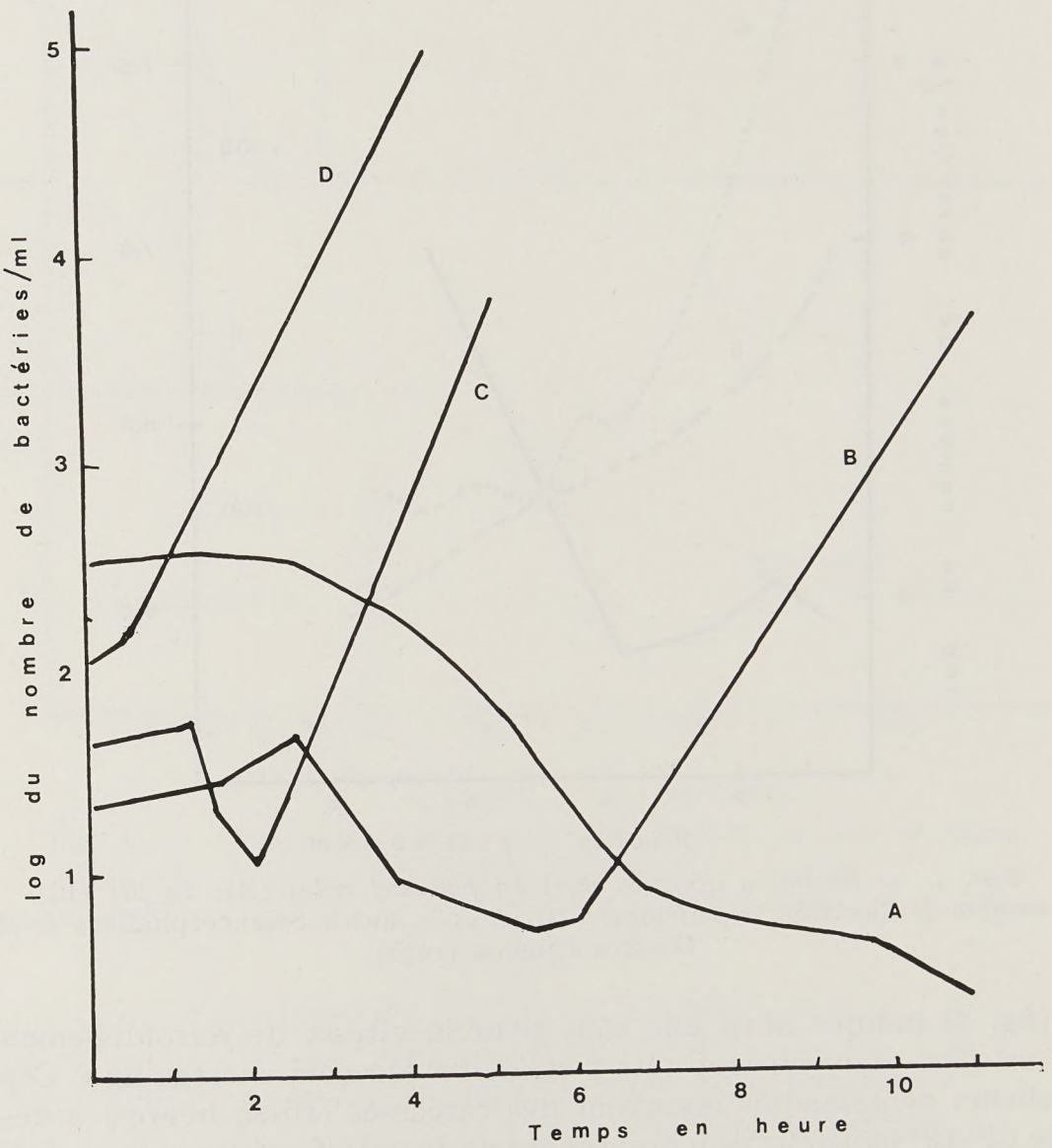

FIG. 3. — *Effect du potentiel redox (Eh) sur la croissance de* Clostridium perfringens *à 37 °C dans du bouillon nutritif à pH 7,0.* D'après INGRAM (1972)

Valeurs de Eh : A) + 230 mV; B) + 215 mV; C) + 115 mV; D) — 35 mV.

Le deuxième facteur qui agit sur la croissance de *Clostridium perfringens* est le pH. Un pH de 7,0 favorise le développement de la bactérie, mais les pH inférieurs ralentissent la vitesse de multiplication. Aussi, au fur et à mesure que l'acidité du milieu augmente, la bactérie voit son temps de génération croître.

Ainsi, si on intègre les 3 facteurs (température, Eh et pH) on observe (fig. 4) sur le *Sternocephalicus* de cheval un début de croissance de *Clostridium perfringens*

à 4 h *post-mortem* c'est-à-dire pour un Eh de — 50 mV ce qui confirme les essais de laboratoire. Cette valeur de — 50 mV apparaît dans le *Longissimus dorsi* à des temps variables de 2 h jusqu'à 24 h. Ingram suggère que le muscle reste avec un potentiel redox élevé pendant les 8-10 premières heures *post-mortem*. Mais parallèlement l'acidité augmente dans la viande mais pas suffisamment vite pour inhiber *Clostridium perfringens* de façon notable. Aussi faut-il se servir du froid.

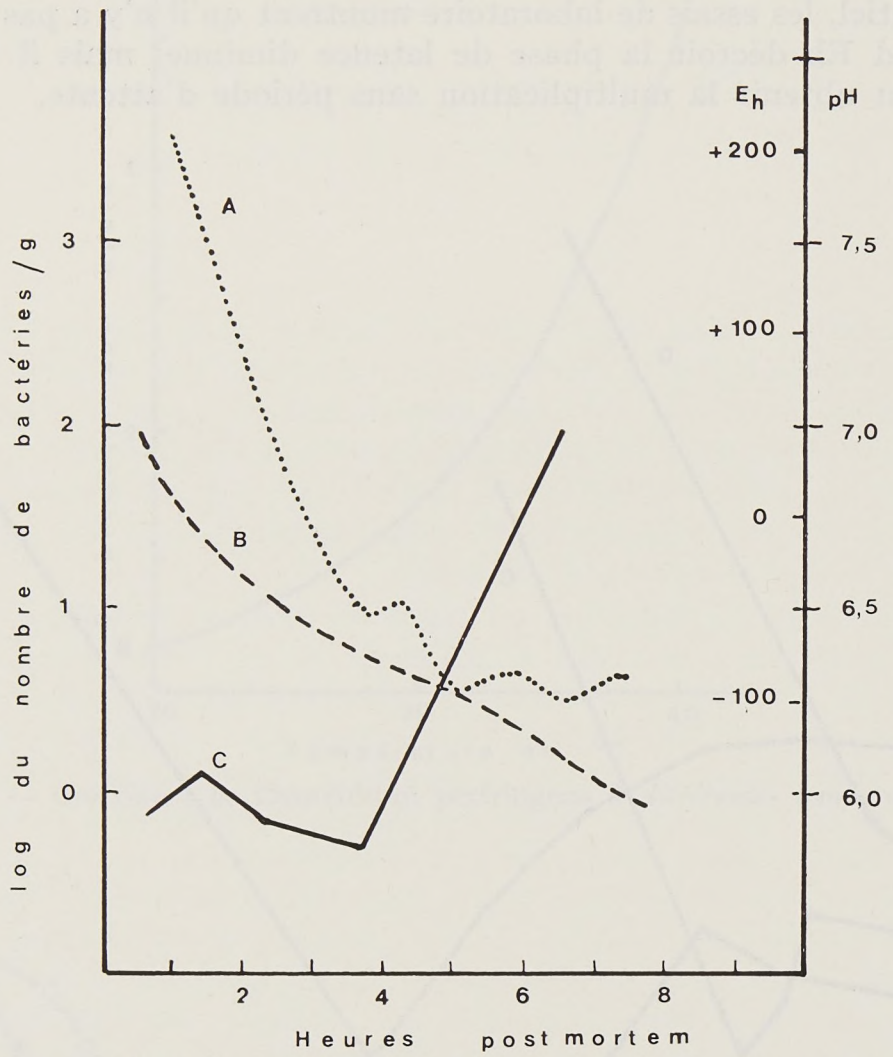

Fig. 4. — *Évolution après la mort du potentiel redox* (*A*), *du pH* (B) *et du nombre de* Clostridium perfringens (*C*) *dans le muscle* Sternocephalicus *de cheval*
D'après Ingram (1972)

Rosset (fig. 5) indique ainsi une zone pour la vitesse de refroidissement. Il ne faut pas, en effet, refroidir trop vite pour éviter le « cold shortening ». Cependant, des chercheurs néozélandais ressuyent des carcasses (16-24 heures) à des températures de 13°-16° ou 18 °C; ils n'observent pas de putréfaction verte profonde après un séjour ultérieur de 3 jours à 3 °C.

Quoiqu'il en soit, la stimulation électrique qui permet une décroissance rapide du pH doit apporter une sécurité de plus contre *Clostridium perfringens* et peut être permettre de refroidir moins rapidement la carcasse.

A côté des facteurs physico-chimiques qui influent sur la multiplication bactérienne existent les relations entre bactéries. Certaines bactéries ralentissent, sans vraiment inhiber, la vitesse de développement des autres bactéries sans que l'on puisse expliquer le phénomène. Ainsi en ensemençant à 10 °C des lames de viande de bœuf avec des couples de bactéries, Gill et Newton (tabl. 1) observent que *Pseudomonas* n'est pas inhibé ni par *Microbacterium* ni par *Enterobacter*. Par

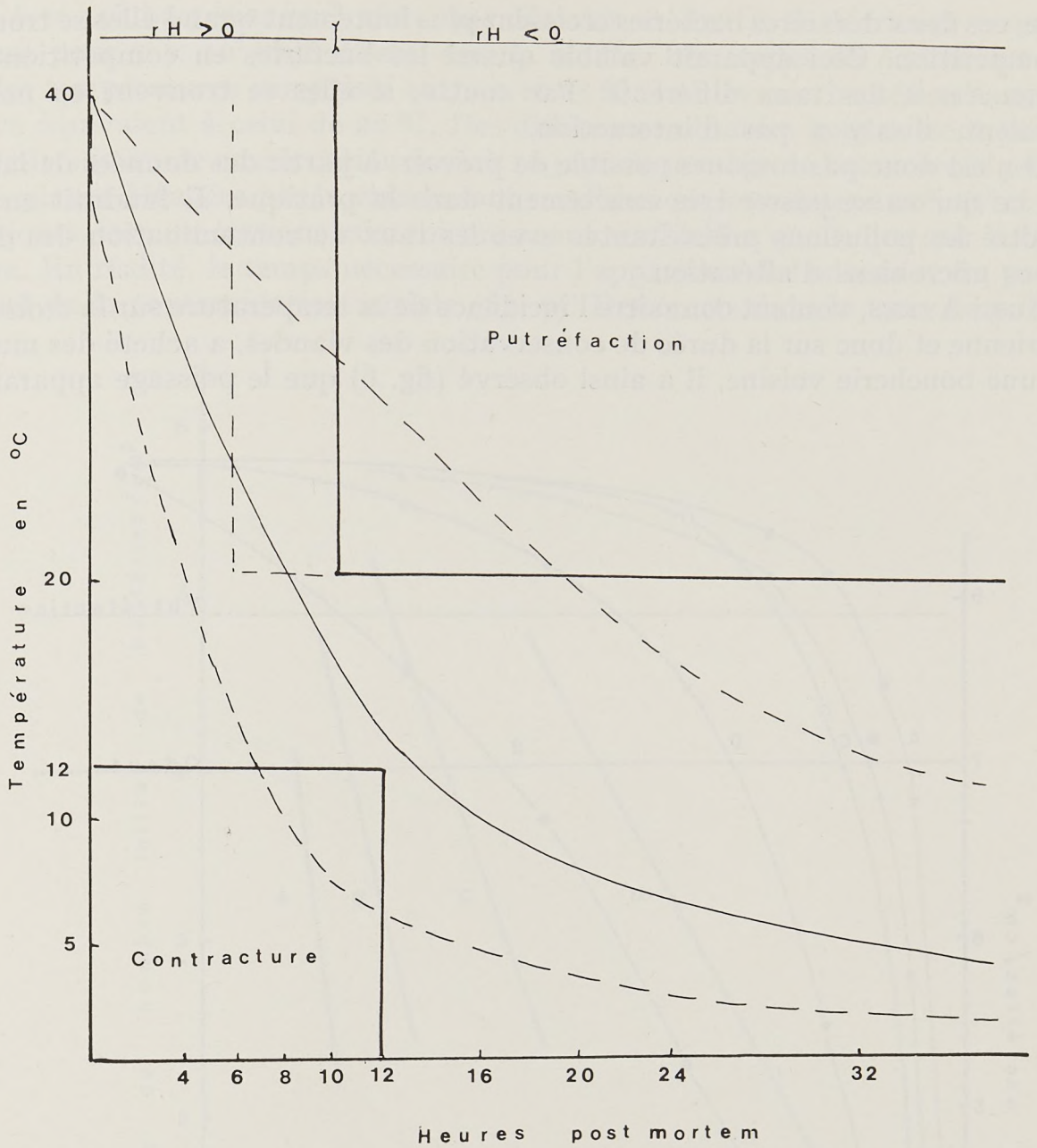

FIG. 5. — *Courbes théoriques de refroidissement de carcasses de bovins*
D'après ROSSET

TABLEAU I

Croissance des bacteries sur la viande à 10 °C
en présence d'autres espèces

Forte inoculation	Faible inoculation	Temps de génération (*h*) 2e espèce	
		seule	mélange
Pseudomonas . .	*Enterobacter* . . .	3,5	10,4
	Microbacterium .	3,4	7,2
Enterobacter . . .	*Pseudomonas* . .	3,2	3,2
	Microbacterium .	3,4	9,1
Microbacterium. .	*Pseudomonas* . .	3,2	3,2
	Enterobacter . . .	3,5	6,0

D'après GILL et NEWTON (1977).

contre, ces deux dernières bactéries croissent plus lentement quand elles se trouvent en compétition. Ceci apparaît valable quand les bactéries en compétition sont ensemencées à des taux différents. Par contre, si elles se trouvent en nombre équivalent, il n'y a pas d'interaction.

Il n'est donc pas toujours possible de prévoir, à partir des données de laboratoire, ce qui va se passer très exactement dans la pratique. Il faudrait en effet connaître les pollutions préexistantes avec les taux de contamination des divers groupes microbiens d'altération.

Aussi AYRES, voulant connaître l'incidence de la température sur la croissance bactérienne et donc sur la durée de conservation des viandes, a acheté des muscles dans une boucherie voisine, il a ainsi observé (fig. 6) que le poissage apparaissait

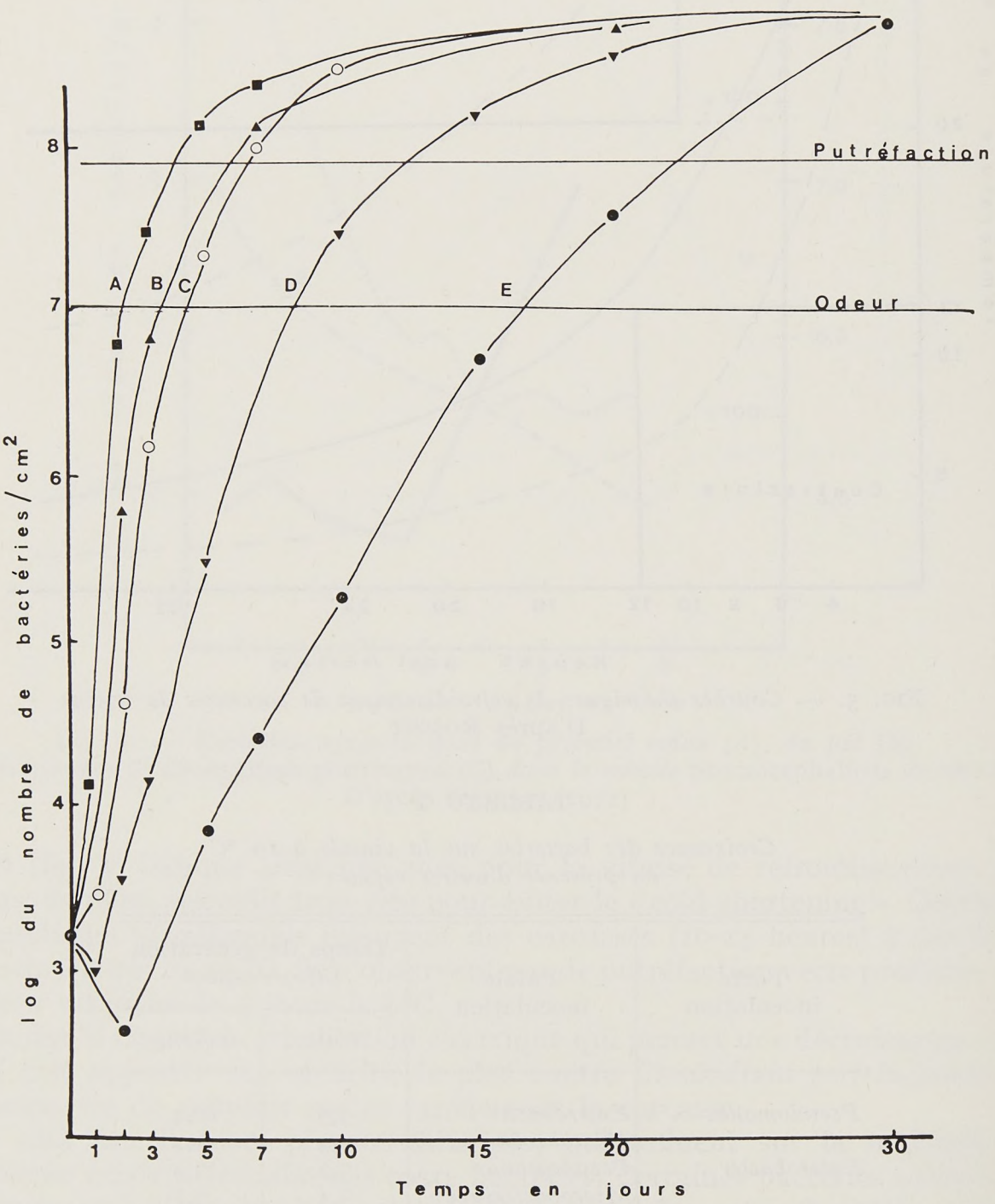

FIG. 6. — *Évolution de la contamination bactérienne en surface de viande bovine en fonction de la température*
D'après AYRES (1960).

A) 20 ou 25 °C; B) 15 °C; C) 10 °C; D) 5 °C; E) 0 °C.

pour 6×10^7 bactéries/cm² alors que HAINES et SMITH le décrivent comme se plaçant pour 3×10^7/cm², c'est-à-dire la moitié. Les études de AYRES ont porté sur des températures comprises entre 0 et 25 °C. Le devenir bactérien à 25 °C se trouve équivalent à celui de 20 °C. Des différences notables apparaissent pour les 5 premiers jours de conservation mais à 10 jours le nombre de bactéries, le poissage et les odeurs dégagées par la viande sont sensiblement les mêmes à 20°, 15° et 10 °C. A 30 jours, les numérations microbiennes sont semblables quelle que soit la température. En réalité, le temps nécessaire pour l'apparition des odeurs et du poissage dépend de la contamination initiale (fig. 7). Des écarts dans le taux de pollution

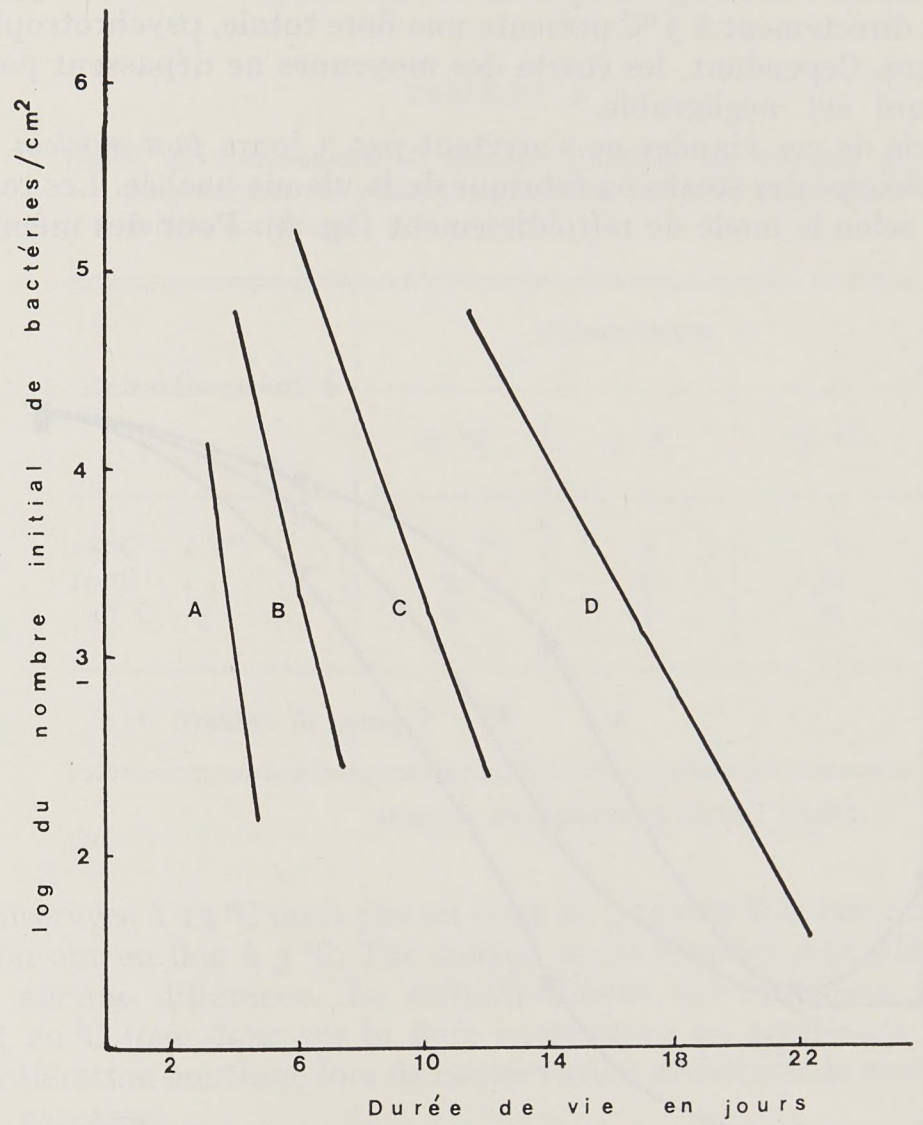

FIG. 7. — *Durée de vie de viande bovine en fonction de la contamination initiale et de la température de conservation*
D'après AYRES.

A) 20 ou 25 °C; B) 10 °C; C) 5 °C; D) 0 °C.

entraînent des variations importantes aux basses températures; mais le phénomène apparaît beaucoup moins sensible aux températures élevées. Ainsi, il n'y a qu'un jour de différence à 20 °C dans la durée de conservation pour des contaminations initiales variant de 1 à 100. Ceci peut s'expliquer par la composition de la flore renfermant proportionnellement moins de bactéries putréfiantes gram — pour des pollutions faibles.

D'après ces résultats de laboratoire les temps nécessaires à l'apparition du poissage apparaissent relativement longs, 2 jours, à 20 °C pour une contamination

moyenne de 10 bactéries /cm². Aussi certains chercheurs ont-ils essayé de refroidir plus lentement les carcasses pour améliorer la tendreté de la viande. Ainsi FIELDS *et al.* placent leurs animaux à des températures comprises entre 14 et 19 °C pendant des périodes de 12-16 ou 20 h *post-mortem* avant de les stocker à 2 °C. De même KASTNER *et al.* font-ils observer des paliers à 10 °C de 6-8 ou 10 h *post-mortem.* Les comparaisons effectuées avec les demi-carcasses refroidies classiquement montrent qu'il n'y a pas de différences entre les numérations effectuées après le palier de température ou après un stockage ultérieur de 2 jours à 2 °C.

Cependant, THOMAS *et al.* notent quant à ceux des écarts bien que leurs demi-carcasses ne soient restées que 8 h à 13 °C avant d'être refroidies 38 h à 3 °C. La viande placée directement à 3 °C présente une flore totale, psychrotrophe ou mésophile, inférieure. Cependant, les écarts des moyennes ne dépassent pas 0,5 en log (3 fois) ce qui est négligeable.

Mais la vie de ces viandes ne s'arrêtent pas 3 jours *post-mortem.* Ainsi, REY *et al.* ont-ils découpé des steaks ou fabriqué de la viande hachée. Les taux de pollution diffèrent selon le mode de refroidissement (fig. 8). Pour des mêmes temps de

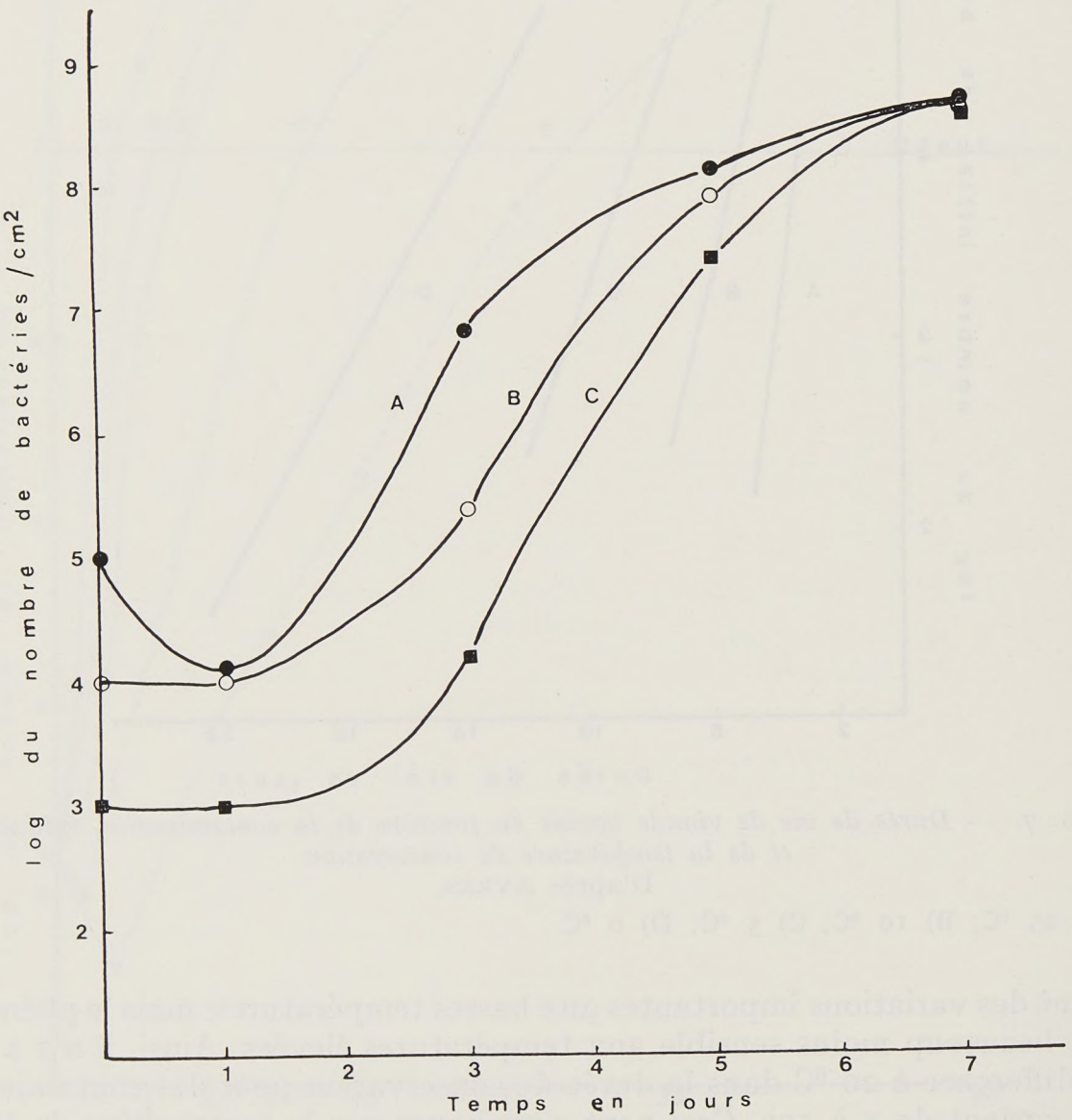

FIG. 8. — *Influence du mode de refroidissement des carcasses bovines sur l'évolution bactérienne en surface de biftecks préparés à partir de ces carcasses et conservés à 5 °C*

Conservation des carcasses :

A) 11 jours à 2 °C; B) 2 jours à 16°-22 °C puis 2 jours à 2 °C; C) 4 jours à 2 °C.

D'après REY *et al.* (1970).

conservation *post-mortem*, les viandes refroidies lentement sont polluées plus que les autres. Une image semblable est obtenue pour les coliformes.

Les résultats obtenus sur l'agneau par PATTERSON et GIBBS (tabl. 2) sont similaires à ceux que nous venons de voir pour le bœuf. Ainsi des gigots refroidis 24 h à 18 °C puis 72 h à 3 °C voient leur flore augmenter de 40 fois alors que la contamination diminue pour ceux placés directement à 3 °C. Il en est de même si le palier de température se situe à 10 °C au lieu de 18 °C mais avec des variations plus faibles. La conservation des viandes refroidies à 18 °C apparaît moins longue que celles mises à 10 °C surtout si les températures de stockage choisies se trouvent élevées. Ainsi les odeurs apparaissent en 2 jours pour les viandes refroidies à

TABLEAU 2

Durée de conservation de viande d'agneau en fonction des températures de refroidissement des carcasses et de conservation des pièces.

Refroidissement à	Conservation à		
	15 °C	7 °C	3 °C
18 °C - 1 (*) . . .	2 (*)	5	7
10 °C - 1	3	6	32
3 ° C - 7	6	12	32

(*) Nombre de jours.

D'après PATTERSON et GIBBS (1978).

18 °C et conservées à 15 °C mais pas au bout de 32 jours si le refroidissement et la conservation ont eu lieu à 3 °C. Par contre, si ces viandes sont placées sous vide il n'existe aucune différence. Le refroidissement des carcasses avec un palier entre 10 et 20 °C joue donc sur la flore bactérienne en accélérant son évolution; et cette accélération continue lors de conservation ultérieure de morceaux prélevés dans cette carcasse.

Cependant, la carcasse paraît relativement protégée puisqu'elle se trouve encore recouverte de gras et d'épimysium (aponévrose). Dans le cas de muscle exposé chaud à l'air la vitesse de croissance apparaît beaucoup plus rapide: des taux de 10^6 *Pseudomonas* /cm², bactérie putréfiante, peuvent être observés au bout de 2 h sur de la viande entreposée en tas dans une pièce à 10-12 °C. Ceci explique pourquoi il est hors de question de désosser de la viande chaude et de lui faire subir telle que un palier de température supérieur à 10 °C pour en assurer la tendreté.

La mise sous vide s'impose donc pour empêcher la croissance des bactéries protéolytiques aérobies et celle de *Microbacterium* qui se multiplie moins vite en l'absence qu'en présence d'air.

Les premiers essais de HEINZ réalisés avec un passage à 15 °C montrent que les lactobacilles se développent de façon importante. Si le temps de séjour est de l'ordre de 12-15 h les entérobactéries, donc peut-être les *Salmonella*, se multiplient

d'environ 10 fois. Aussi pour éviter tout risque cet auteur recommande-t-il de réduire la durée du palier à 5-8 h.

Cependant, si on passe par une température de 10°C, au lieu de 15 °C, on peut augmenter, sans risque, le temps de séjour jusqu'à 36 h. Dans ces conditions les croissances bactériennes prennent place pendant les premiers jours de conservation (fig. 9B) en particulier les lactobacilles favorisés par la mise sous vide et par le CO_2 provenant de la respiration du muscle. Par contre, sur les viandes conditionnées après ressuyage (fig. 9A), les bactéries restent en phase de latence pendant les premiers jours. Au 28e jour, les numérations des viandes désossées à chaud et des viandes désossées après refroidissement se trouvent voisines; une seule exception, les entérobactéries qui se multiplient environ 100 fois sur les premières et 3 fois sur les secondes.

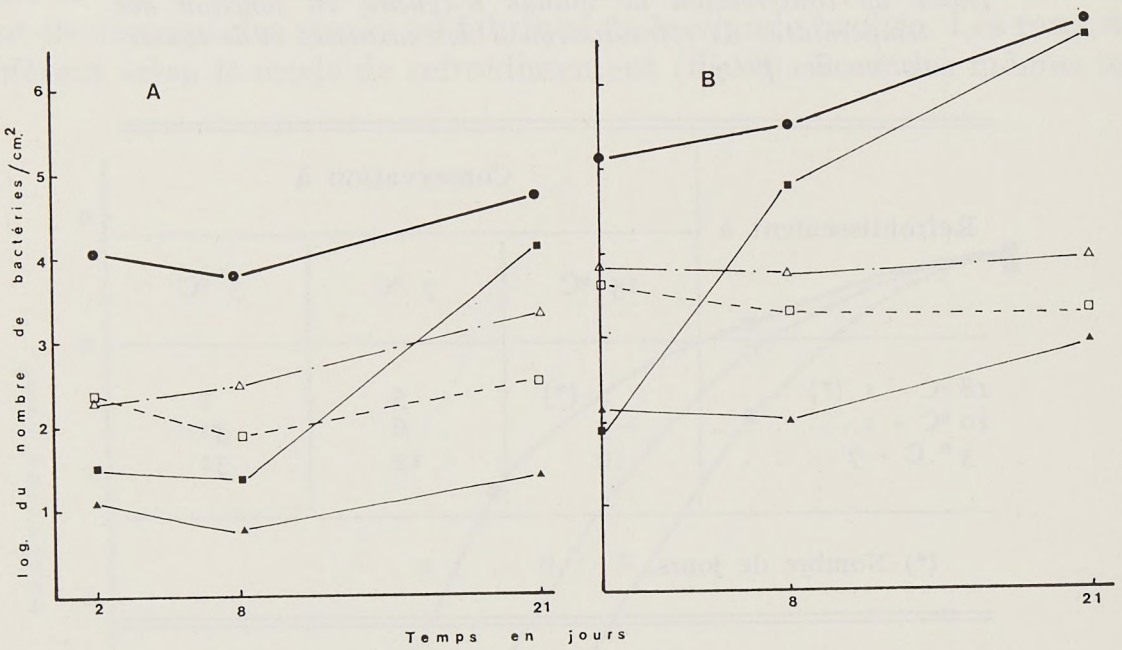

FIG. 9. — *Évolution bactérienne en surface de pièces de viande bovine désossées après ressuyage (A) ou désossées à chaud (B) au cours d'une conservation sous vide. Température de conservation 0-2 °C pour A ; 10 °C pendant 36 h puis 0-2 °C pour B.*

■———■ *Lactobacillus* □— — —□ *Microbacterium* △— . —△ *Pseudomonas*
▲———▲ enterobactéries ●———● flore totale à 22 °C.

Au point de vue bactériologique, l'utilisation de ces viandes désossées à chaud ne pose pas de problème. Reprises en barquette les viandes de moins de 14 jours en sous vide montrent une croissance moindre que sur les viandes témoin compensant ainsi la plus forte multiplication en sous vide. Après les 14 premiers jours en sous vide, les évolutions paraissent semblables. De même, reprises sous atmosphères contrôlées CO_2-O_2 ces viandes, d'après des travaux exposés par BUCHTER, évoluent comme les viandes témoin de même âge *post-mortem ;* les conclusions semblent identiques pour les viandes hachées.

Et la stimulation électrique?

Tous les auteurs s'accordent à dire que la stimulation électrique n'a pas d'influence sur les bactéries : aucune destruction, ni inhibition, ni stimulation.

En conclusion, les bactéries se développent en effet plus rapidement sur les viandes chaudes que sur les viandes placées à basse température. Toutefois l'utilisation de la technologie du sous vide qui entraîne une modification des équilibres bactériens permet de maintenir la viande à des températures supérieures à 10 °C

sans risque de dégradation pour cette dernière. Il ne faut pas non plus tomber dans l'excès inverse c'est-à-dire dépasser 15 °C à cause de la multiplication possible de *Clostridium perfringens* et donc de l'apparition de la putréfaction verte.

La stimulation électrique qui augmente rapidement l'acidité du milieu viande et accélère l'apparition de la tendreté doit permettre de fournir au consommateur, toutes choses égales par ailleurs, une viande de meilleure qualité bactériologique.

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Les impératifs électriques de la stimulation

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Parmi les divers stimulis capables de provoquer la contraction artificielle des muscles, l'électricité est le plus utilisé, notamment parce qu'il est très facile d'en contrôler à la fois l'intensité et la durée. Au niveau de l'application technologique de la stimulation électrique aux différentes carcasses, il a été établi (BENDALL, 1980, HOULIER, 1980) que pour des temps après saignée de l'ordre de 20 à 40 mn, le champ électrique au niveau de chaque muscle était un paramètre déterminant dans l'efficacité de la stimulation. Il est important de rappeler que du point de vue de la technologie des viandes, le seul critère de l'efficacité de la stimulation est l'accélération constatée, instantanément et à court terme, de la chute du pH. C'est un critère beaucoup plus restrictif que celui des physiologistes qui prennent généralement en compte simplement le mouvement. En pratique, cela signifie que l'on ne peut pas juger de l'efficacité d'une stimulation sur le degré apparent de contraction de la musculature. Par exemple, sur une carcasse de mouton, la stimulation d'un seul muscle, par un système approprié d'électrodes, déclenche de violentes contractions de l'ensemble de la musculature, mais seul le muscle directement stimulé montre une baisse de pH.

Dans ces conditions, il est clair que, au moins pour la technique dite « haute tension » c'est-à-dire celle appliquée après éviscération et dépouille, la distribution du champ électrique dans la carcasse conditionne l'efficacité de la stimulation au niveau de chaque muscle. Nous allons examiner les différents facteurs qui déterminent cette distribution.

I. — Distribution du champ électrique dans une carcasse

Au cours de la stimulation, le courant électrique circule dans un circuit qui comprend trois parties (fig. 1) :

— la partie générateur d'impulsion et conducteurs électriques qui inclue en général un élément de rail, un chariot et un crochet servant d'électrode, l'autre électrode étant constituée et appliquée de façon variable selon les matériels;

— la carcasse proprement dite;

— l'interface électrode carcasse.

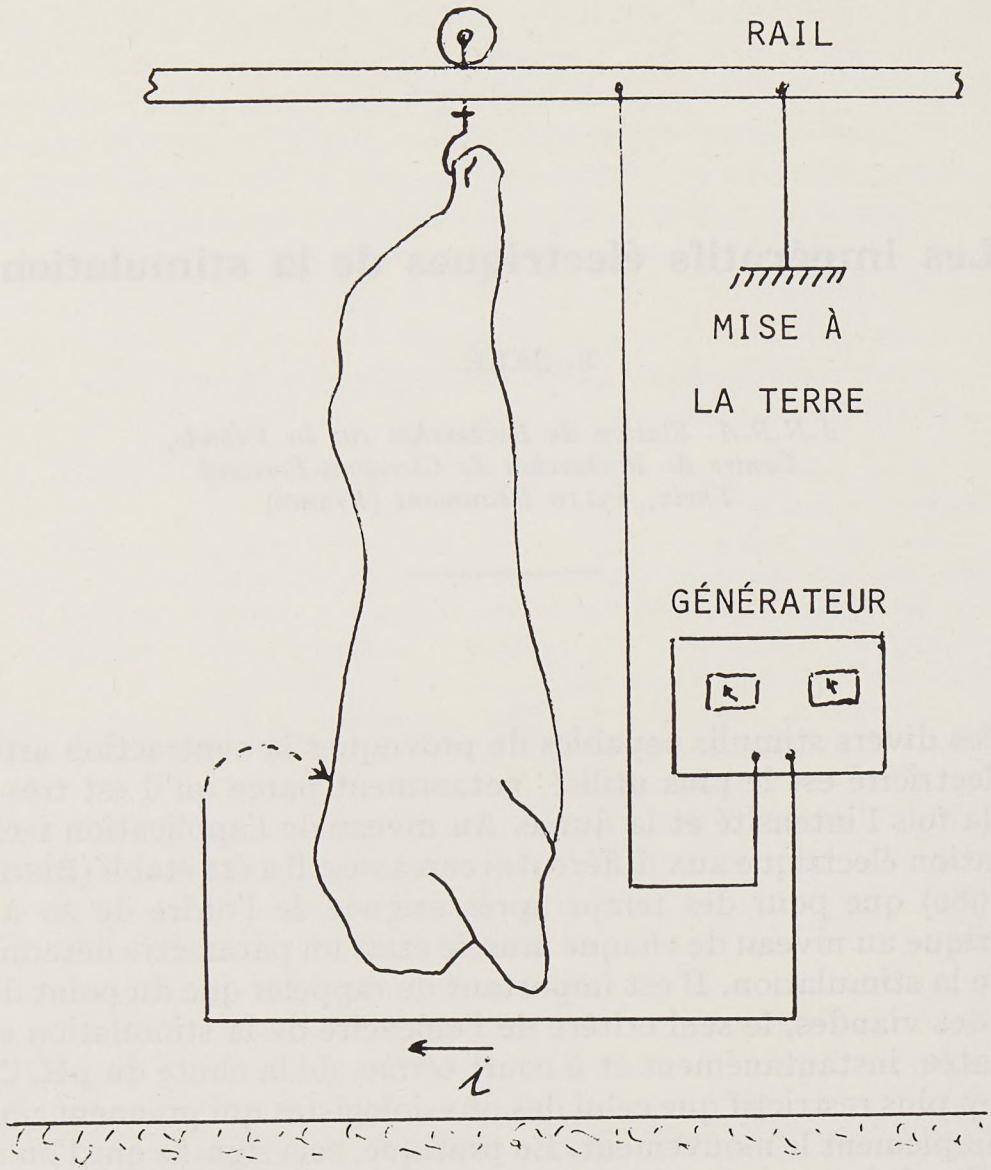
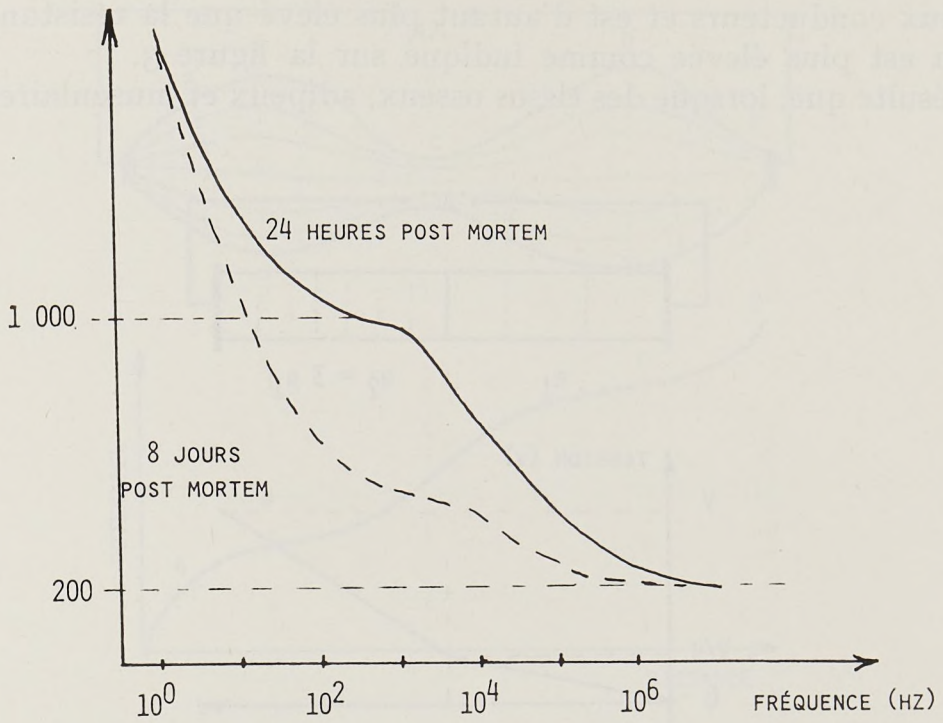


FIG. 1. — *Circuit de stimulation haute tension.*

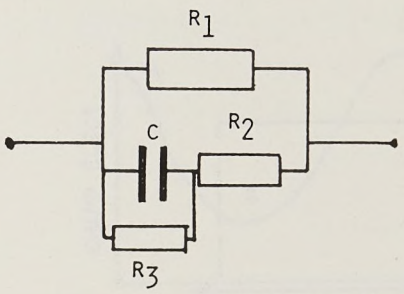
Dans les deux dernières parties, carcasse et interface, la circulation du courant électrique met en jeu des phénomènes spécifiques.

1.1. — *Conduction dans la carcasse*

Dans les différents tissus qui constituent la carcasse, le courant est transporté par les ions, principalement les ions Cl^- et K^+ au niveau des masses musculaires. La « résistance » électrique de ces tissus dépend du nombre d'ions présents dans le milieu et de la facilité avec laquelle ils peuvent se déplacer, c'est-à-dire de la structure fine du milieu. Dans le cas des tissus biologiques et en particulier pour le muscle, on observe qu'un courant alternatif simusoïdal circule d'autant plus facilement que sa fréquence est élevée (fig. 2). L'analyse de ce phénomène montre l'existence d'effets capacitifs dont l'origine résulte des propriétés diélectriques particulières des membranes cellulaires et de leur proche environnement. On en déduit que dans le cas d'une impulsion, une vitesse de variation élevée de la tension entraîne à la fois une diminution de la résistance apparente de la carcasse et permet une propagation de l'impulsion dans toute la masse du tissu, les membranes cellulaires présentant alors une faible impédance.



Impédance du tissu musculaire



R_1 = Résistance extracellulaire
R_2 = Résistance intracellulaire
C = Capacité de membrane
R_3 = Résistance de membrane

Schéma électrique équivalent

FIG. 2. — *Conduction du courant électrique dans le tissu musculaire.*

Pour une impulsion donnée, la résistance spécifique du milieu dépend de la nature des tissus traversés. Le tissu osseux est le plus résistant, puis les tissus adipeux et conjonctifs; enfin le tissu musculaire est le plus conducteur, et il est plus conducteur dans la direction des fibres que dans la direction perpendiculaire aux fibres. Ces différences de résistance au passage du courant électrique sont à l'origine de variations du champ électrique dans la carcasse.

1.2. — *Hétérogénéité du champ électrique due aux différences de résistance électrique*

Rappelons d'abord que le champ électrique représente le taux de variation du potentiel avec la distance. C'est une grandeur vectorielle, définie par sa direction et son intensité, exprimée en volts par mètre ou en volts par centimètre.

Dans un conducteur homogène et de géométrie simple, par exemple un cylindre, le champ est constant et simplement égal ou quotient de la différence de potentiel entre les électrodes par la distance des électrodes.

Lorsque deux conducteurs de géométrie semblables mais de résistance spécifique différente sont placés en série, le champ présente des valeurs différentes

dans les deux conducteurs et est d'autant plus élevé que la résistance spécifique d'un milieu est plus élevée comme indiqué sur la figure 3.

Il en résulte que, lorsque des tissus osseux, adipeux et musculaires se trouvent

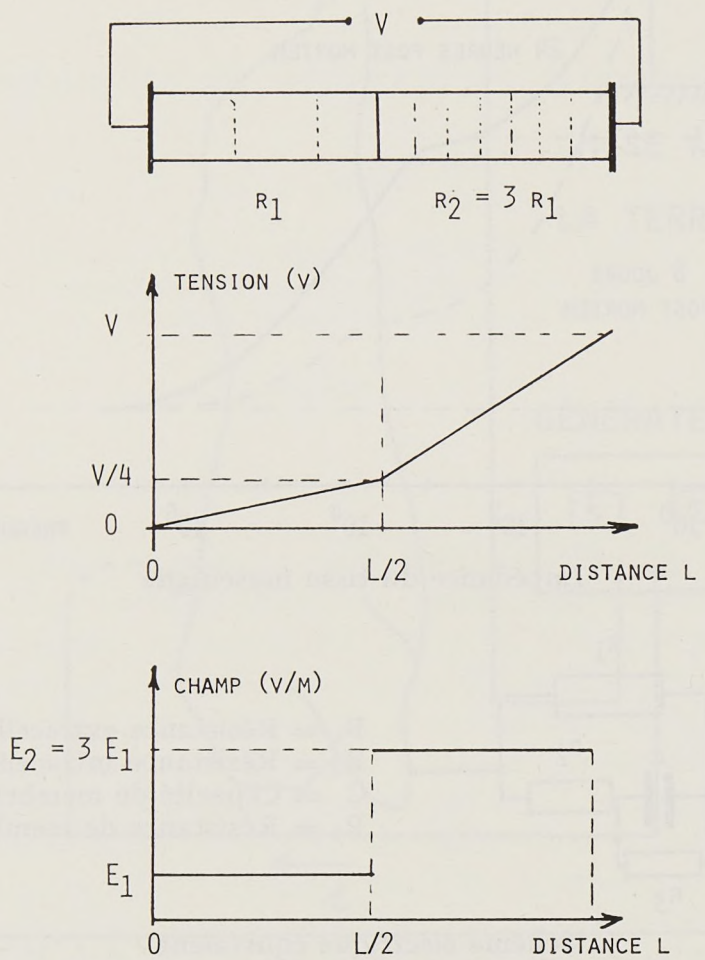


FIG. 3. — *Variations du champ dues aux différences de résistivité.*

placés en série dans une géométrie voisine de celle d'un cylindre, le champ est maximum dans les tissus osseux et minimum dans les tissus musculaires.

Par contre, placés en parallèle, ces corps sont tous soumis au même champ.

1.3. — *Hétérogénéité de forme*

Même dans le cas d'un conducteur homogène, le champ électrique n'est uniforme que pour des géométries très simples et d'une façon générale il n'est pas possible de calculer sa distribution.

Si nous considérons un corps ayant l'allure de celui représenté sur la figure 4, on voit sur le premier graphique que le potentiel le long de ce corps varie rapidement dans les zones de faible section, c'est-à-dire au voisinage des électrodes et dans la région centrale. C'est aussi dans ces zones que le champ est le plus élevé.

En déplaçant les électrodes, on modifie évidemment de façon très importante cette distribution du champ. Si, par exemple, on déplace une électrode de *a* en *c*, ceci revient, en pratique, à annuler le champ électrique dans toute la partie *ac* du corps. Il en résulte que la position des électrodes joue un rôle déterminant dans la distribution du champ dans la carcasse.

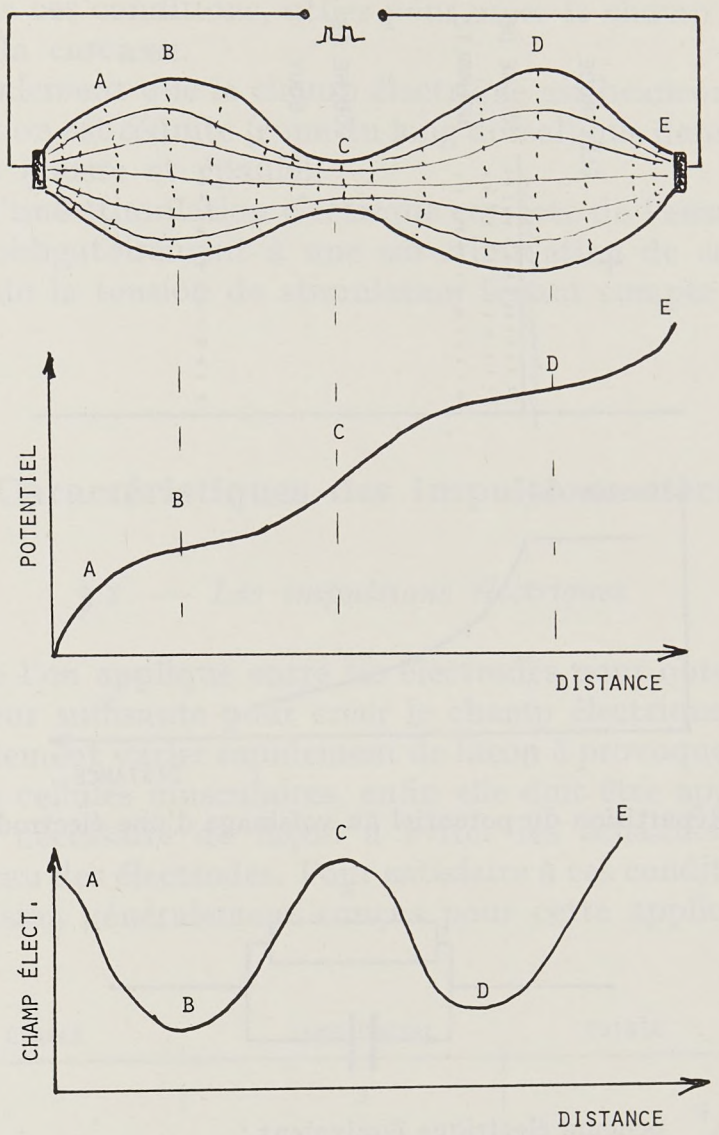


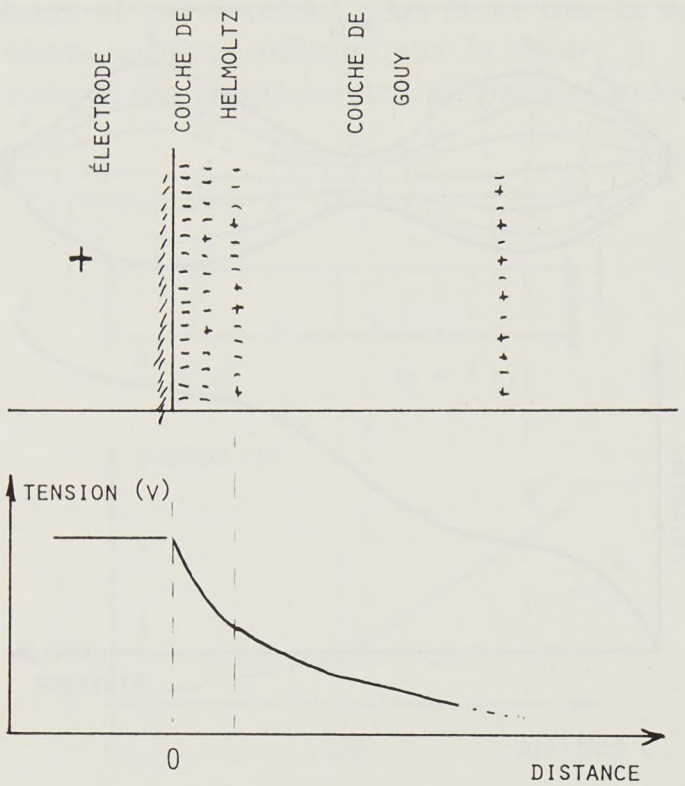
FIG. 4. — *Influence de la géométrie du corps sur la distribution du champ électrique.*

II. — Les phénomènes d'interface aux électrodes

2.1. — *L'interface électrode-carcasse*

Le passage d'un régime de conduction électronique dans les conducteurs métalliques, à un régime de conduction ionique s'effectue à la surface des électrodes. Cette surface se recouvre d'un film liquide et les forces électrostatiques qui agissent sur les ions en solution dans ce film en fonction de leur charge électrique produisent une distribution telle que celle indiquée sur la figure 5. Si la différence de potentiel électrode /film est suffisante (en pratique supérieur à 2 volts) et maintenue au cours du temps, la surface est le siège d'un phénomène d'électrolyse avec production de Cl^2 et KOH qui entraînent par réaction secondaire des dégradations des tissus et des électrodes et qui se traduisent en plus par l'augmentation de la résistance électrique entre électrode et tissus.

Pour s'affranchir dans une très large mesure de ces inconvénients, il suffit d'inverser la polarité des électrodes avec une fréquence suffisante, supérieure à environ 20 Hz dans le cas d'un courant sinusoïdal. Du point de vue électrique, l'inter-



Répartition du potentiel au voisinage d'une électrode

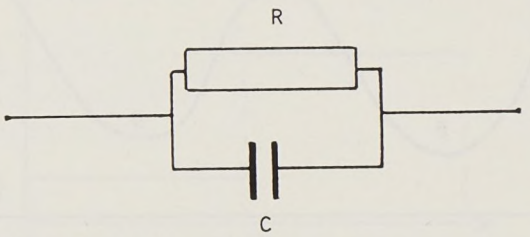


Schéma électrique équivalent :

R : Résistance de contact
C : Capacité des couches de Helmoltz

FIG. 5. — *Phénomènes d'interface métal /muscle.*

face est alors équivalente au circuit représenté sur la figure 5. Comme la valeur de la capacité, qui correspond à la couche de Hel moltz peut être très élevée, ce circuit offre une faible impédance aux impulsions de courant.

2.2. — *Conséquences pratiques*

Les différences dans la géométrie et la composition des différentes parties de la carcasse d'une part, les phénomènes d'interface d'autre part, se combinent pour modifier l'intensité et la distribution du champ électrique au niveau des différents muscles.

Cet effet est illustré sur la figure 6 qui représente la distribution du potentiel et celle du champ électrique dans une carcasse de mouton, les électrodes étant placées à l'extrêmité des pattes. On remarque la très brutale variation du potentiel au voisinage de ces électrodes, variations dues aux effets cumulés de la polarisation et de la faible section. Il en résulte que seuls 40 p. 100 environ de la tension totale

appliquée sont, dans ces conditions, utiles pour créer le champ électrique dans la majeure partie de la carcasse.

On constate également que le champ électrique est beaucoup plus élevé dans les parties où la section est réduite (zone du long dorsal) que dans les volumes musculaires importants (cuisse et épaule).

La recherche d'une stimulation électrique correcte de l'ensemble de la musculature conduira obligatoirement à une sur-stimulation de certains muscles et une augmentation de la tension de stimulation tenant compte des conséquences de la polarisation.

III. — Caractéristiques des impulsions électriques

3.1. — *Les impulsions électriques*

La tension que l'on applique entre les électrodes pour obtenir la stimulation doit avoir une valeur suffisante pour créer le champ électrique désiré en chaque point. Elle doit également varier rapidement de façon à provoquer la dépolarisation des membranes des cellules musculaires, enfin elle doit être appliquée pendant le temps strictement nécessaire de façon à éviter les échauffements indésirables notamment au niveau des électrodes. Pour satisfaire à ces conditions, on utilise des générateurs de tension généralement conçus pour cette application, et dont les

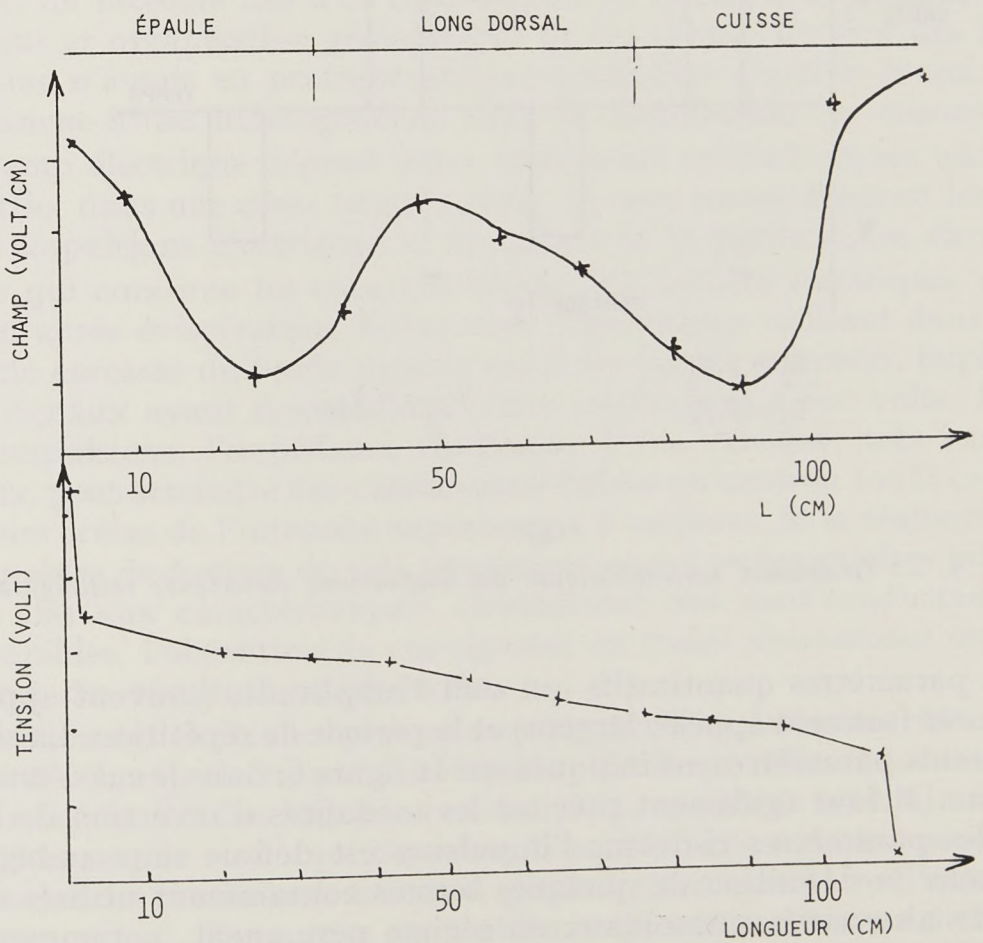
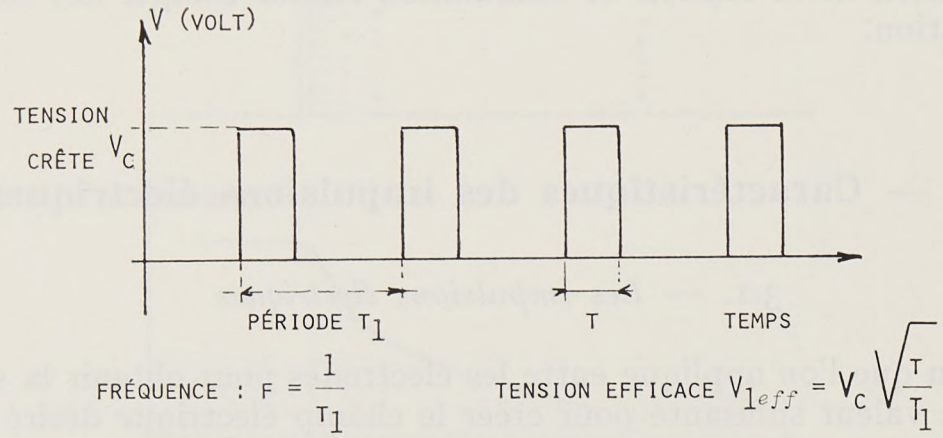


FIG. 6. — *Distribution de la tension et du champ électrique sur une carcasse de mouton* (d'après HOULIER 1980).

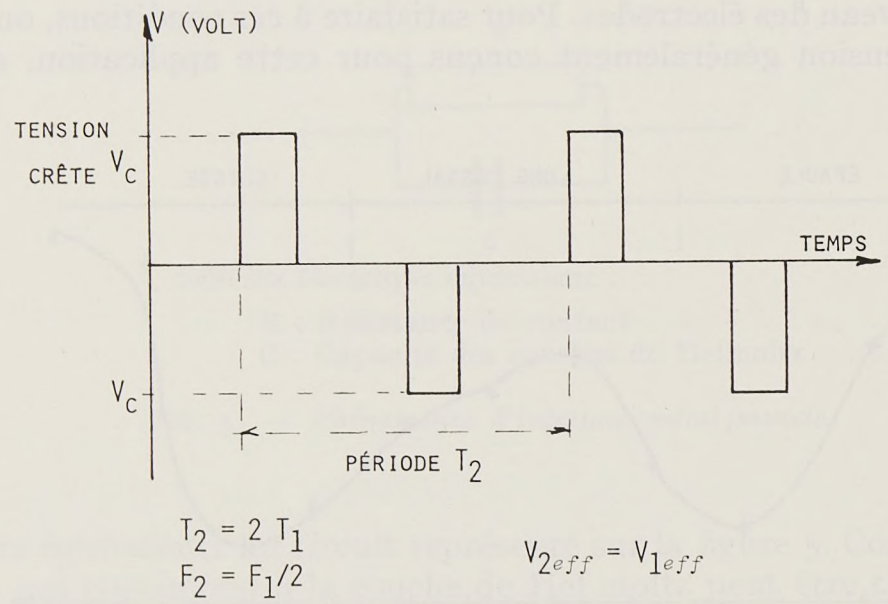
caractéristiques peuvent être très différentes. Il apparaît ici utile de rappeler quelques définitions relatives aux impulsions électriques.

Une impulsion électrique est définie par un ensemble de paramètres qui sont :

1) la loi de variation de la tension (ou de l'intensité) en fonction du temps. Ce paramètre souvent quanlitatif définit la forme de l'impulsion : les formes les plus utilisées sont les impulsions rectangulaires, triangulaires, sinusoïdales.



(A)



(B)

FIG. 7. — *Grandeurs caractéristiques des impulsions électriques rectangulaires.*

2) des paramètres quantitatifs qui sont l'amplitude, (souvent appelée valeur crête), la durée (souvent appelée largeur) et la période de répétition. La signification de ces différents paramètres est indiquée sur la figure 6, dans le cas d'une impulsion rectangulaire. Il faut également préciser les modalités d'invertion de la polarité.

Avec les paramètres ci-dessus l'impulsion est définie sans ambiguïté. Nous allons rappeler la définition de quelques termes couramment utilisés dans le cas des courants alternatifs sinusoïdaux en régime permanent, notamment pour les courants industriels. Dans ce cas, on définit sans ambiguïté, la période, c'est-à-dire l'intervalle de temps au bout duquel le signal se répète de façon identique,

et la fréquence, égale à l'inverse de la période. L'application de ces définitions au cas des courants de stimulation ne semble pas avoir été faite de façon correcte; les deux signaux de la figure 7 par exemple ne présentent pas la même fréquence au point de vue électrique mais chaque impulsion provoquant une contraction, on aura tendance à leur attribuer la même fréquence.

La notion de valeur efficace est également très courante pour les courants périodiques. La valeur efficace (RMS en anglais) correspond à la valeur équivalente d'un courant continu qui produirait le même effet thermique pendant une période. Cette notion très utile en électrotechnique n'a pas de sens en physiologie. En effet un signal rectangulaire ayant une tension crête de 1 000 Volts appliqué pendant 1 milli-seconde toutes les secondes à une valeur efficace de 31,6 volts.

Enfin, il ne faut pas oublier que la plupart des appareils de mesure électrique sont calibrés pour mesurer des valeurs en courant continu ou des valeurs efficaces sur des courants sinusoïdaux, uniquement, et dans certaines limites de fréquence.

La seule façon correcte d'étudier et de mesurer les caractéristiques des impulsions fournies par un générateur pour stimulation électrique des carcasses est d'observer ces impulsions sur un écran d'oscilloscope.

Conclusion

L'obtention en un point déterminé de la carcasse d'un champ électrique suffisant pour obtenir une stimulation convenable du muscle dépend pour une part importante, de facteurs liés à la constitution de la carcasse (composition en différents tissus et organisation géométrique de ces tissus). Ce sont des facteurs sur lesquels nous n'avons en pratique aucune possibilité d'action et qui conduisent obligatoirement à des hétérogénéités dans la distribution du champ électrique.

Le champ électrique dépend aussi de facteurs technologiques que nous pouvons maîtriser dans une assez large mesure : ce sont essentiellement les caractéristiques des impulsions électriques et la nature et la position des électrodes.

En ce qui concerne les caractéristiques des signaux électriques et pour une application après éviscération, l'obtention d'un champ suffisant dans la majeure partie d'une carcasse de bovin excitée entre les points extrêmes, implique l'utilisation de signaux ayant des tensions crêtes supérieures à 600 volts. Par ailleurs, pour ces impulsions, l'impédance électrique d'une carcasse, très variable selon les animaux, peut atteindre des valeurs aussi faibles qu'environ 100 Ω ce qui conduit à des valeurs crêtes de l'intensité supérieure à 6 ampères. Si la réalisation de générateurs capables de fournir de tels signaux en ondes rectangulaires pose quelques problèmes liés aux caractéristiques insuffisantes des semi-conducteurs actuellement disponibles, l'obtention de ces signaux en ondes sinusoïdales est plus facile et conduit à des résultats satisfaisants.

La position des électrodes permet aussi de modifier de façon très importante la répartition du champ électrique. La situation idéale est celle où les électrodes sont placées aux extrêmités des zones à stimuler. Cette condition est toujours à peu près correctement remplie en ce qui concerne la moitié arrière de la carcasse, l'extrémité des membres postérieurs recevant généralement une paire d'électrodes (crochets de suspension). Par contre un problème apparaît au niveau de l'avant où il est difficile d'éviter les zones de champ nul. Par exemple l'application d'une électrode au niveau du cou ne permettra pas une stimulation correcte de la patte avant

et de l'épaule et réciproquement. Ces difficultés ne pourront être résolues que par l'utilisation de dispositifs multi-électrodes dont la mise en œuvre semble actuellement délicate.

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Stimulation électrique et désossage à chaud : Quelques résultats français

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Résumé

La stimulation électrique est apparue à de nombreux chercheurs comme une solution possible en vue de résoudre les problèmes de dureté de la viande liés aux phénomènes de contraction musculaire, que l'on rencontre notamment dans le cas d'un désossage à chaud ou d'une réfrigération très rapide des carcasses après la mort.

Les travaux réalisés par l'ITEB en abattoir sur carcasses de veaux et gros bovins avec un stimulateur haute tension confirment l'intérêt de la stimulation, pour faire chuter rapidement le pH des carcasses.

Dans le cas d'un désossage à chaud suivi d'un refroidissement immédiat des muscles à 0 /+ 2 °C la stimulation permet d'éviter en grande partie le durcissement de la viande normalement observé.

Cependant pour véritablement éliminer toute dureté résiduelle il faudra sans doute, en plus de la stimulation, prévoir pour les muscles désossées à chaud un palier de température à + 10 °C pendant quelques heures avant de les placer à 0 /+ 2 °C. L'intérêt du désossage à chaud pour la réduction des pertes au cours du stockage a pu également être vérifié.

Appliquée à des carcasses devant subir une réfrigération modérée (+ 6 °C /24 h) et un désossage classique à froid la stimulation électrique n'améliore pas la tendreté au terme d'une durée de maturation normale. On peut dans ce cas au plus espérer une certaine accélération de la maturation mais il reste à préciser l'importance de cette accélération, qui n'aurait d'ailleurs vraiment d'intérêt que pour des filières intégrant abattage, découpe et distribution.

Cadre de l'étude

Il n'existe pas à ce jour en France d'installations utilisant couramment le désossage à chaud et (ou) la stimulation électrique des carcasses. Toutefois des essais ont eu lieu, d'autres sont en cours dans un certain nombre d'entreprises sous l'égide des pouvoirs publics et en collaboration avec les Centres de Recherche.

Le présent article se propose de rapporter les principales conclusions d'es-

sais réalisés par le Laboratoire Viande de l'ITEB (1) dans le cadre d'une action concertée de la DGRST (2).

Nous ne reviendrons pas en détail sur les raisons qui ont conduit un certain nombre de chercheurs de par le monde à proposer l'application aux carcasses encore pantelantes d'une stimulation électrique. Pour simplifier disons que cette technique est apparue comme une solution possible en vue de résoudre les problèmes de dureté de la viande liés essentiellement au phénomène de cold shortening.

Ce cold shortening (ou cryochoc) correspond à une contraction des fibres musculaires entrainant une dureté irréversible de la viande, contraction qui risque d'apparaître chaque fois que l'on soumet la musculature avant l'installation de la rigidité cadavérique à un froid trop intense, et plus particulièrement lorsque les muscles sont séparés de leur support osseux ce qui leur laisse toute possibilité de se contracter (cas des muscles désossés à chaud).

Le cold shortening tend à se produire au moins dans trois cas de figure correspondant à des évolutions récentes qui apparaissent dans la transformation des viandes :

— réfrigération rapide (réfrigération-choc) des carcasses après l'abattage,
— congélation des carcasses *ante-rigor* (agneaux essentiellement),
— désossage à chaud (*ante-rigor*) suivi d'un refroidissement immédiat des muscles conditionnés sous-vide.

Certains auteurs, surtout Américains, allant au delà des objectifs que nous venons d'évoquer, ont envisagé d'appliquer la stimulation électrique également dans le cas d'une réfrigération classique des carcasses en vue d'accélérer la maturation de la viande, voire même d'offrir au consommateur une viande plus tendre, toutes choses égales par ailleurs.

Les résultats présentés dans ce document devraient permettre de mesurer l'intérêt technique d'une stimulation électrique appliquée d'une part dans le cas d'une réfrigération classique, d'autre part dans le cas d'un désossage à chaud suivi d'un refroidissement immédiat des muscles; et ce pour des carcasses de type Français à savoir des vaches de réforme et des veaux de boucherie, ce dernier type d'animal n'ayant pratiquement pas été étudié car il n'est guère utilisé ailleurs qu'en Europe.

Dispositif expérimental

- Réalisé en vrai grandeur dans un abattoir public (Villers-Bocage — Calvados), cet essai respectait le dispositif suivant :

  — une première série de carcasses dont on ne retenait que les demies gauches constituait les *Témoins :*

  Abattage traditionnel, suivi d'un ressuage de 24 heures à + 6 °C.

  Ensuite passage en chambre de conservation à 0 / + 2 °C.

  Désossage et mise sous-vide (Cryovac BB_1) à 48 heures *post-mortem* et conservation des muscles sous-vide à 0 / + 2 °C jusqu'à 5 jours *post-mortem* pour les veaux (11 j *post-mortem* pour les vaches).

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— une deuxième série de carcasses de caractéristiques identiques aux témoins était stimulée électriquement après l'éviscération et avant la fente (haut voltage) :

Les demies droites (stimulés désossés à froid) suivaient ensuite exactement le même processus que les témoins.
Les demies gauches étaient désossées à chaud entre 1 h 30 et 2 heures *post-mortem* (*stimulés désossés à chaud*) ; les muscles ainsi prélevés étaient aussitôt mis sous-vide (Cryovac BB_1) et conservés à 0/+ 2 °C jusqu'à la même date que les témoins et les stimulés désossés à froid soit 5 jours *post-mortem* pour les veaux (11 j *post-mortem* pour les vaches). A l'ouverture des sacs sous-vide étaient réalisés les différents contrôles de qualité de viande, de plus pour chaque muscle retenu une tranche de viande était mise en barquette et conservée une semaine à 0/+ 2 °C pour suivre la durée de vie résiduelle du produit.

- Dans chaque lot les effectifs étaient de 20 et comprenaient par moitié des sujets de race Normande et de race FFPN. Le poids moyen des carcasses froides était de 114 kg pour les veaux témoins et de 113,5 kg pour les veaux stimulés; pour les vaches le poids moyen des carcasses des témoins était de 293 kg et celui des carcasses stimulées de 294 kg. Les caractéristiques des carcasses ont été appréciées par rapport aux définitions de référence de la classification officielle française. Pour les veaux, le niveau moyen de conformation était intermédiaire entre celui de la classe O et de la classe R, et pour les vaches il correspondait à le classe O. L'état d'engraissement moyen était celui de la classe 3 pour les veaux et pour les vaches. La classe moyenne de couleur de la viande des veaux était intermédiaire entre la classe 2 (rosé clair) et la classe 3 (rosé).
- Les muscles étudiés ont été les muscles *Longissimus dorsi* (LD), *Semitendinosus* (ST), *Triceps branchii caput longum* (GA) et *Rectus abdominis* (DA). Les valeurs figurant dans les tableaux 1, 3, 5 et 6 correspondent aux moyennes des mesures effectuées sur ces 4 muscles.

Stimulation électrique (Caractéristiques du matériel utilisé)

Le procédé utilisé faisait appel à des tensions élevées, et comportait 4 électrodes (2 par pole) fixées aux quatre pattes de la carcasse dépouillée mais non fendue.

Moment de la stimulation	Veaux 14 mn *post-morten* Vaches 25 mn *post-mortem*
Durée de stimulation	3 mn
Tension de crête	Veaux 420 V Vaches 710 V
Durée de l'impulsion	10 millisecondes
Fréquence	Une impulsion toutes les 80 millisecondes (12,5 HZ)
Inversion de polarité	Toutes les 5 secondes

Résultats

Chute de pH

La stimulation appliquée aux carcasses entraîne dans tous les cas une importante chute de pH par rapport aux témoins, mais sans que le pH ultime 48 heures après la mort soit modifié (tabl. 1).

TABLEAU 1

pH 1 h 10 post-mortem, *à l'entrée en réfrigération*

	Témoins	Stimulés	Écart
Veaux	6,82	5,94	— 0,92
Vaches	6,79	6,03	— 0,76

Température à cœur

A l'entrée en réfrigération (1 h 10 *post-mortem*) on note des températures à cœur légèrement plus élevées (1 à 2 °C) dans les carcasses stimulées, notamment au niveau du *Longissimus dorsi* (tabl. 2).

TABLEAU 2

Température à cœur (7 cm) à 1 h 10 post-mortem (°C)

		Témoins	Stimulés	Écarts
Veaux	LD . .	33,7	35,9	+ 2,2
	ST . .	39,3	39,9	+ 0,6
Vaches	LD . .	34,4	36,8	+ 2,4
	ST . .	38,5	39,5	+ 1,0

Pertes au ressuage

Les pertes de poids en carcasse pendant les premières 24 heures ne sont pas différentes entre témoins et stimulés (1,8 p. 100 en veau, 1,65 p. 100 en vaches). Dans le cas des demies carcasses stimulées puis désossées à chaud, en dehors des pertes par évaporation pendant l'opération de désossage proprement dite et qui de toute façon restent faibles, il n'y a pas de pertes au ressuage puisque les muscles désossés à chaud sont aussitôt conditionnés sous-vide (film Cryovac BB_1).

Exsudat à la sortie du sous-vide (1)

Les pourcentages moyens d'exsudat observés selon les traitements sont indiqués au tableau 3.

TABLEAU 3

Exsudat à la sortie du sous-vide

(en % du poids mis sous-vide)	Témoins	Stimulés	
		Désossés froid	Désossés à chaud
Veaux . . . . . (jours de sous-vide)	0,22 (3 jours)	0,46 (3 jours)	0,63 (5 jours)
Vaches . . . . . (jours de sous-vide)	0,30 (9 jours)	0,39 (9 jours)	0,54 (11 jours)

On note que les taux d'exsudat sont faibles dans l'ensemble (du fait d'une température de 0/+ 2 °C strictement respectée) avec une tendance à ce que les muscles des carcasses stimulées puis désossées à froid après un ressuage classique exsudent un peu plus que leurs homologues non stimulées (témoins).

La stimulation suivie d'un désossage à chaud donne des taux d'exsudat plus élevés, mais rappelons d'une part que ces muscles sont restés globalement 2 jours de plus en sous-vide, et d'autre part que cet exsudat constitue pratiquement la seule perte de poids subie par ces carcasses puisqu'elles échappent aux pertes au ressuage.

Aspect commercial des muscles

Il est intéressant de noter qu'avant l'ouverture des sacs un jugement visuel de l'aspect commercial des muscles a été réalisé. Ce jugement était nettement favorable aux muscles stimulés puis désossés à chaud, jugés moins exsudatifs (surface des muscles plus sèche à l'œil), plus compacts et de consistance plus ferme que ceux des 2 autres traitements (témoin et stimulés /désossés à froid). Ceci aussi bien en veau qu'en gros bovin. L'aspect moins exsudatif des muscles désossés à chaud peut sembler paradoxal par rapport aux chiffres du paragraphe précédent.

Après l'ouverture des sacs, les jugements visuels restent également favorables aux muscles désossés à chaud plus rebondis et dont la graisse apparait plus ferme et plus blanche. Alors que sur les témoins et sur les stimulés désossés à froid, la graisse, du moins dans le cas des muscles de vache, apparait plus jaune (avec quelques reflets verdâtres) et la consistance du muscle plus molle.

L'aspect plus rebondi des muscles désossés à chaud traduit le fait que ces muscles ont subi une contraction musculaire importante (malgré la stimulation) et qu'ayant ainsi perdu de la longueur, ils ont gagné en « forme ».

Ce phénomène est particulièrement net dans le cas du *Semitendinosus* (rond de gîte) pour lequel on a pu mesurer la longueur totale du muscle (tabl. 4).

TABLEAU 4

Longueur totale du muscle Semitendinosus *à la sortie du sous-vide (cm)*

	Stimulés	Stimulés
	Désossés à froid (1/2 droite)	Désossés à chaud (1/2 gauche)
Veaux . . .	29,0	26,0
Vaches . . .	40,0	37,0

A noter qu'à la coupe les muscles désossés à chaud présentent également un aspect plus attractif et notamment un grain de viande apparemment plus fin mettant mieux le persillé en valeur.

Couleur de la viande

Que ce soit à l'abattoir ou sur les muscles à la sortie du sous-vide, par jugement visuel ou méthode instrumentale, on n'observe pas de différence significative pour la couleur entre les trois traitements étudiés. Ce aussi bien en veau qu'en gros bovin (tout au plus une légère tendance à ce que les muscles désossés à chaud paraissent légèrement plus brillant en surface — mais ceci n'est pas lié à la stimulation).

De plus des barquettes confectionnées à la sortie du sous-vide ont été conservées à une température stricte de 0/+ 2 °C pendant une semaine. Au terme de ce délai les barquettes de veau avaient toujours bel aspect et l'on n'observait aucune différence entre traitements.

Les barquettes de bœuf présentaient quelques auréoles brunes (metmyoglobine), mais là encore aucune différence entre les 3 traitements.

Pertes à la cuisson

Les chiffres moyens observés sont indiqués dans le tableau 5.

TABLEAU 5

Pourcentage de pertes à la cuisson
(type escalope pour le veau, grillade pour le bœuf — Méthodologie ITEB)

	Témoins	Stimulés	
		Désossés à froid	Désossés à chaud
Veaux	20,1	21,6	19,9
Vaches	14,7	15,6	14,1

Pour les 2 types d'animaux dans le cas d'un désossage à froid après ressuage classique, la stimulation tend à augmenter les pertes à la cuisson (comme pour l'exsudat précédemment). Par contre la stimulation associée au désossage à chaud limite ces mêmes pertes.

Tendreté après maturation
(5 j *post-mortem* pour les veaux, 11 j pour les vaches)

Les mesures mécaniques réalisées par cisaillement à l'aide de l'appareil de Salé sur viande cuite ne traduisent pas d'écart significatif entre traitements pour le veau, et sont légèrement défavorable au désossage à chaud pour les vaches (tabl. 6).

TABLEAU 6

Travail de cisaillement (W) sur viande cuite mesuré par l'appareil de SALÉ *(en millijoule)*

	Témoins	Stimulés	
		Désossés à froid	Désossés à chaud
Veaux	184	174	183
Vaches	229	248	262

Le jury de dégustation, qui reste encore la meilleure référence pour mesurer l'acceptabilité d'une viande vue sous l'angle du consommateur, n'a pas décelé de différence de tendreté entre témoins et stimulés /désossés à froid, par contre il a émis un avis défavorable sur les muscles désossés à chaud, dans le cas du veau comme du bœuf (cf. tabl. 7). Cependant le jury n'a pas décelé de différences quant à la saveur et la jutosité.

TABLEAU 7

Tendreté appréciée par le jury (fréquence des réponses par note de tendreté)

Type d'animal	Notation de la tendreté	Témoins	Stimulés (désossés à froid)	Stimulés (désossés à chaud)
Vaches	Très dur ou dur	20	26	31
	Acceptable	34	26	46
	Tendre ou très tendre . .	46	48	23
Veaux	Très dur ou dur	18	13	28
	Acceptable	19	28	41
	Tendre ou très tendre . . .	63	59	31

Conclusions

- Soulignons d'abord *l'efficacité de la stimulation que nous avons appliquée dans cet essai en terme de chute de pH* (1 unité pH pour les veaux, 0,8 pour les gros bovins à l'entrée en réfrigération).

  Concernant la stimulation proprement dite, pour les procédés utilisant les hautes tensions, les paramètres essentiels nous paraissent être *la tension de crête, le délai d'intervention après la mort* et *la durée d'excitation.* Il ne parait pas utile de rechercher systématiquement des tensions très élevées : 300/400 V en veaux et 600/700 V en gros bovins semblent suffire. Il resterait toutefois à dresser une cartographie de la répartition du champ électrique dans l'ensemble de la musculature. On doit intervenir le plus tôt possible après la mort mais les délais sont moins contraignants qu'avec les procédés Basse Tension. La durée de stimulation demande à être mieux étudiée afin de déterminer le temps minimum nécessaire (probablement de l'ordre de 1 mn 30 à 2 mn) compatible avec les impératifs d'une chaîne d'abattage.

  L'inversion de polarité des impulsions, par séquence comme dans notre étude, ou par l'utilisation d'un courant alternatif est à recommander. Enfin on a intérêt à utiliser *quatre électrodes* (dont 2 constituées par les crochets des pattes AR) pour une meilleure répartition du champ électrique dans la carcasse car les effets de la stimulation varient selon les muscles considérés, et sont en particulier plus marqués au niveau du Long dorsal (faux-filet en gros bovins) en accord avec les résultats des travaux Américains.

- Le stimulateur proprement dit en haute tension ne coûte que 15 000 F environ (Stimulateur basse tension : environ 1 500 F) plus les aménagements de sécurité évidemment indispensables, et les modifications de chaine dans le cas d'installations existantes.

- Les chutes de pH enregistrées indiquent-elles que la stimulation a été efficace à 100 p. 100, c'est-à-dire qu'on a évité tout risque de contracture des fibres musculaires?

  Les modifications de forme et de longueur des muscles désossés à chaud montrent que ce n'est pas le cas, il y a eu une contraction, certes limitée, mais entrainant une dureté accrue perçue par le jury de dégustation. Attention par conséquent, *une chute de pH même prononcée n'élimine pas tout risque de contracture,* car on n'arrive pas en fait à détruire tout l'ATP du muscle, et même avec la stimulation il faudra rester prudent sur l'utilisation du froid.

- Cela dit la stimulation a incontestablement produit *un effet bénéfique dans le cas du désossage à chaud suivi d'un refroidissement immédiat.* Si l'on rapproche les résultats obtenus avec ceux d'essais antérieurs effectués par notre laboratoire on constate que la stimulation a notablement réduit l'écart de dureté qu'entrainerait un désossage à chaud avec refroidissement immédiat des muscles à 0/+ 2 °C.

  On obtient ainsi des résultats comparables à ceux que l'on observe lorsqu'on maintient les muscles à + 10 °C pendant 24 à 36 heures après le prélèvement à chaud (en sachant que dans la pratique cela représenterait un délai trop long pour satisfaire aux exigences des hygiénistes) (tabl. 8).

TABLEAU 8

Écarts en p. 100 par rapport à des carcasses réfrigérées classiquement (24 h à + 6 °C) et désossées à froid (48 h post-mortem)

	Désossage à chaud		
	+ Refroidissement immédiat à 0/+ 2 °C (ITEB résultats antérieurs) (%)	+ Stimulation + Refroidissement immédiat à 0/+2 °C (%)	+ Palier à + 10°C pendant 24/36 h avant mise à 0/+ 2 °C (ITEB résultats) antérieurs) (%)
W Cuit (travail de cisaillement sur appareil de SALE)	+ 45	+ 14	+ 10
Note tendreté (Jury) . . .	— 42	— 12	— 8

Il n'en reste pas moins que, malgré la stimulation, la qualité de viande sous l'angle de la tendreté a été un peu dégradée. Soulignons cependant que les muscles ont subi dans cet essai le traitement le plus détériorateur qu'on puisse imaginer. Pour éviter toute dureté résiduelle on devrait pouvoir trouver un moyen terme, soit en repoussant le moment du désossage par rapport à la mort, avec le risque que la stimulation entraîne une rigor précoce, soit en combinant la stimulation avec un palier de quelques heures seulement à + 10 °C après désossage à chaud et mise sous vide, ce qui n'empêchera aucunement d'amener ensuite les muscles aux 7 °C à cœur réglementaires moins de 24 heures *post-mortem*.

- La stimulation *appliquée dans le cas d'un ressuage classique* apporte-t-elle un avantage?

  Il est clair qu'à 5 jours de maturation en veau et 10 jours en gros bovins *la stimulation n'apporte rien en tendreté*, et par contre amène une certaine détérioration en exsudat et pertes cuisson. D'autres travaux ont montré que la stimulation dans le cas d'un ressuage classique accélère la maturation mais il reste à préciser (en fonction du type d'animal) l'importance de cette accélération dont les effets sont en tout état de cause effacés après 5 jours en veau et 10 jours en gros bovins.

  Cette accélération de maturation n'aura d'ailleurs vraiment d'intérêt que pour des chaines intégrant découpe et distribution car il ne faut quand même pas espérer que 2 ou 3 jours de maturation puissent suffire (délai de vente de nombreux grossistes industriels).

- Quelques mots enfin sur les autres aspects :

  Contrairement aux allégations de certains auteurs la stimulation électrique ne semble modifier ni la saveur ni la jutosité. Pas d'effet non plus sur la couleur après maturation et la durée de vie résiduelle de la viande en barquettes.

- Sur le plan de l'aspect commercial des muscles et surtout de la réduction des pertes en cours de stockage (et même à la cuisson) se confirme une fois de plus

l'avantage du désossage à chaud. Notons que c'est bien le désossage à chaud en lui-même et non la stimulation électrique qui apporte cet effet bénéfique.

- Des contrôles bactériologiques ont été réalisés mais n'ont pu être à ce jour dépouillés. Les premiers résultats ajoutés à des observations antérieures nous amènent à réaffirmer que le désossage à chaud bien conduit ne détériore pas, voire améliore, la qualité bactériologique des viandes, et que de toute façon les problèmes bactériologiques sont à traiter à l'abattage c'est-à-dire au moment de la contamination et non *a posteriori*.

  La stimulation en elle-même n'intervient pas apparemment sur la bactériologie

En conclusion la stimulation est-elle un gadget?

Certainement pas lorsqu'on l'applique dans les conditions où elle peut agir c'est-à-dire essentiellement dans le cas d'un désossage à chaud, d'une réfrigération choc ou d'une congélation ante-rigor.

Et pourtant certains la conçoive comme un gadget devant servir d'argument publicitaire, c'est regrettable car cela risque de déconsidérer un procédé à bien des titres prometteurs.

D'autres ont recours à la stimulation pour des problèmes de classement des carcasses, mais cet aspect de la question d'une part n'apporte rien à la qualité finale de la viande vue sous l'angle du consommateur, d'autre part ne concerne pas les pays Européens compte-tenu de leurs propres méthodes de classement.

Enfin ceux qui prétendent dans l'absolu pouvoir grâce à la stimulation garantir une viande plus tendre, avouent implicitement qu'ils ne savaient pas précédemment conduire leur maturation.

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Electrical Stimulation of Bull carcasses: Use of different voltages and relation of tenderisation to protein fragmentation

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Electrical stimulation (ES) of beef carcasses or sides shortly after slaughtering has been studied extensively and is commercially introduced in many countries (for review see e.g. BENDALL, 1980). Since 1977 E.S. has been studied in our laboratory (VANDEKERCKHOVE *et al.*, 1978) (VANDEKERCKHOVE and DEMEYER, 1978) (CASTEELS *et al.*, 1979) involving a total of 36 animals. The top half of A.C. was used giving pulsed D.C. of 50 Hz (pulse width 10 m-sec.) at 100-150 V_{RMS} equivalent to 200-300 V_{max}

($V_{RMS} = V_{max} \times \sqrt{\frac{q}{200}}$ with q = pulse frequency) and average currents (RMS) of 100 mA, 200 mA and 600 mA, applied consecutively for 80 sec.

Stimulated and control sides were cooled (2° C) immediately after E.S. (expts. 1, 2, 3 and 4) or first conditioned for 22 h at 15 °C (expt. 5). Measurements of shear force after heating (JOSEPH, 1977) and sarcomere length were carried out on *longissimus* and *latissimus dorsi* muscles, sampled from one (VERBEKE and VAN DE VOORDE, 1978) or three-rib (MARTIN and TORREELE, 1962) cuts, obtained after cooling (2 °C) for at least 1 day.

Table 1 summarizes the results obtained, showing that bull carcass sides, cooled immediately after slaughter (expts. 1, 2 and 3) have more tender meat after E.S. The shear force of the muscles sampled was lowered by 16 ± 5 p. 100 (mean value ± S.E.) due to E.S. The tenderizing effect seems related to the prevention of cold-shortening as indicated by the longer sarcomeres in stimulated muscles (table 1). Also, when control sides are conditoned to prevent cold-shortening (expt. 5), no tenderization due to E.S. is observed, when average values are considered. When individual values are considered however, data suggest that E.S. has some tenderizing effect, not related to the prevention of cold-shortening (VANDEKERCKHOVE and DEMEYER, 1978). In one experiment, (expt. 4) using heifers, having very tender meat, no effect of E.S. was observed. The possible

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TABLE I

Effect of Electrical Stimulation on Shear Force (kg) and Sarcomere length (μ) (Mean values ± S.E.)

Expt. (1)	Shear Force (kg)				Sarcomere Length (μ)			
	Long. Dorsi		*Lat. Dorsi*		*Long. Dorsi*		*Lat. Dorsi*	
	stimulated	control	stimulated	control	stimulated	control	stimulated	control
1 (16) (2) . . .	5.25 (***) ± 0.72	7.35 ± 0.40	—	—	—	—	—	—
2 (6)	5.43 (*) ± 0.60	6.25 ± 0.52	5.10 ± 0.40	5.43 ± 0.26	2.05 (***) ± 0.05	1.83 ± 0.03	2.95 (*) ± 0.05	1.97 ± 0.26
3 (1)	4.0 (***) ± 1.0	5.2 ± 1.2	5.9 (***) ± 0.8	6.3 ± 0.8	1.99 (***) ± 0.23	1.71 ± 0.22	2.94 (***) ± 0.24	2.04 ± 0.29
4 (9) (3) . . .	3.7 ± 0.4	3.3 ± 0.3	—	—	1.85 ± 0.08	1.97 ± 0.10	—	—
5 (4) (4) . . .	5.12 ± 0.25	5.38 ± 0.60	4.17 ± 0.74	4.58 ± 0.54	1.90 ± 0.09	1.83 ± 0.08	2.96 ± 0.23	2.77 ± 0.14

(1) ( ) = number of animals involved.
(2) The right side of 8 animals was stimulated, the right side of the other 8 animals was used as control.
(3) Heifers were used.
(4) Both sides were conditioned for 22 h at ca. 15 °C.

(*) $p \leqslant 0.05$.
(**) $p \leqslant 0.01$.
(***) $p \leqslant 0.001$.

reasons for this have been discussed (CASTEELS *et al.*, 1979) including less than optimal conditions of E.S. The latter, accoording to BENDALL (1980) include a peak voltage of 600 V, pulsed between 15 and 25 pps for 1.5-2.0 min. (equivalent to V_{RMS} = 164-212 V).

A more thorough study of the effect of E.S. under our conditions, involving sampling of 20 different muscles was described recently (DEMEYER *et al.*, 1980), showing an even distribution of the tenderizing effect over all muscles studied, when carcasses were boned after cooling. Some evidence of a less homogenous tenderization after hot boning was obtained. The same experements also showed that the over-all tenderizing effect obtained (ca. 6 p. 100) was not associated with the prevention of cold-shortening. The latter experiments were completed and are reported here as a comparison between our initial conditions of E.S. and a peak voltage of 600 V as recommended by BENDALL (1980).

Evidence was obtained for accelerated protein fragmentation due to E.S. using sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS — PAGE).

Experimental

All experiments were done using 8 bulls (mean ± S.E. for live weight : 435 ± 15 kg; dressing p. 100 : 60.6 ± 1.0 p. 100; age: 11.6 ± 0.2 months) of Belgian breeds. Within a period of 6 months these animals were slaughtered as described earlier (DEMEYER *et al.*, 1980). The left carcass sides of the first three bulls were stimulated using the apparatus described earlier (VANDEKERCKHOVE *et al.*, 1978) and the conditions described in the introduction.

With the following three bulls pulsed D.C. of the same frequency and pulse width but at 320 -340 V_{RMS} was used. (V_{max} 640-680 V). I_{max} was kept constant at 3 A for 4 min. (I_{RMS} = 1.5 A).

A laboratory constructed microprocessor controlled stimulator, equipped with an oscilloscope and allowing changes of pulse frequency was used. The last 2 bulls were used as blanks, to compare left and right carcass sides without E.S. All carcass sides were cooled at ca. 2 °C immediately after slaughtering and E.S. Temperature and pH was measured at regular intervals after slaughtering on 6 different sites of the carcasses, as described earlier (DEMEYER *et al.*, 1980). After cooling for 1 week, 20 muscles were sampled and used for determination of sarcomere length by laser diffraction and shear force as described earlier (DEMEYER *et al.*, 1980).

In five experiments, two involving the low voltage E.S. and hot boning reported earlier (expts. 3 and 4 in DEMEYER *et al.*, 1980) and the three higher voltage E.S. experiments, protein changes were followed using SDS-PAGE. Immediately after E.S. a sample of longissimus dorsi (corresponding to the 7th and 8th thoracic vertebrae) was removed and separated along the fibre direction into 4 subsamples. Samples were wrapped in alumina foil and conditioned at room temperature for 24 h. They were then vacuum packed (DEMEYER *et al.*, 1980) and cooled at 2 °C. After 0, 2, 7 and 13 days samples (ca. 10 g) were used to prepare protein extracts, which were separated by SDS-PAGE following a modification of the method described by WEBER and OSBORN (1969).

Densitometry was used to quantitative relative protein concentration. The method will be described in detail elsewhere (VANDENDRIESSCHE, CLAEYS, VANDEKERCKHOVE and DEMEYER, in preparation).

TABLE 2

Effect of E.S. on carcass temperature (T_{ES}) and rate of cooling (b)

ES_{100} [2]				ES_{300} [2]				No ES			
Left (E.S.)		Right		Left (E.S.)		Right		Left		Right	
T_{ES} [1]	b [1]	T_{ES}	b	T_{ES}	b	T_{ES}	b	T_{ES}	b	T_{ES}	b
38.4	0.094	35.6	0.093	38.1	0.099	36.2	0.095	38.2	0.099	37.4	0.096
38.8	0.095	37.0	0.087	37.3	0.105	38.0	0.105	38.4	0.097	38.1	0.101
38.0	0.086	37.4	0.086	39.0	0.098	38.0	0.097				

[1] T_{ES} and *b* calculated as described under experimental.
[2] ES_{100} = lower voltage stimulation.
ES_{300} = higher voltage stimulation.

Presentation and treatment of data

The effect of E.S. on shear force and sarcomere length was graphically evaluated by plotting the ratios ES/control of shear force against sarcomere length for all muscles studied (DEMEYER *et al.*, 1980). In an attempt to evaluate the over-all effect of E.S. on the whole carcass side, weighed average shear force values were calculated, using weight factors derived from the physical weight and commercial value of the 20 muscles sampled. (DEMEYER *et al.*, 1980). The effect of ES on rates of cooling and pH fall was assessed by calculation of mean values for the 6 carcass sites measured at each measuring time. Rates were then estimated by regression of these means against time. For both temperature and pH regression following $y = a.e^{-bx}$ was used with $y = {}^0C$ or pH and $x =$ hours after slaughtering (max. 8 h). These equations were then used to estimate the temperature immediately after ES (T_{ES}) ($x = 0.75$ h in $y = a.e^{-bx}$) and the time after stunning at which pH 6 is reached (x value for $y = 6.0$ in $y = a.e.^{-bx}$).

Results and discussion

1. — *Rate of cooling*

From table 2 it can be calculated that E.S. increases carcass temperature by 1.2 ± 0.5 °C (mean value ± S.E.). The difference is significant at the level $p \leqslant 0.1$ when means are compared for all stimulated sides with all non stimulated sides. Paired t-statistics for E.S. experiments only increases significance level to $p \leqslant 0.05$. Over-all rates of cooling are not significantly affected by E.S. and no difference between the effect of lower or higher voltage ES is apparent. It should be mentioned here that, although over-all rates of cooling are not affected by E.S., rates in individual muscles may be affected.

2. — *Rate of pH decline*

Table 3 shows that E.S. accelerates the drop of pH. Where control sides take 6.1 ± 0.4 h (mean value ± S.E.) to reach pH 6, ES lowers this time to 3.2 ± 0.4 h. Lower voltage seems to be more efficient in this accelerating effect than higher voltage. These results are similar to the values obtained by BENDALL *et al.* (1976) using 700 V_{max} and by BOUTON *et al.* (1980) using 110 V_{max}.

3. *Shear force and Sarcomere length*

In two experiments without E.S. the mean ratio Left/right for shear force values for all muscles sampled was 0.96 ± 0.12 (mean value ± S.D. for 20 muscles in duplicate). This large variation is illustrated in fig. 1, also showing the variation in the ratio for sarcomere length Left/right. In spite of this variation, close inspection of figure 1 shows that E.S. tends to shift the shear force ratio to lower values, indicating tenderisation. This shift however is not associated with longer

TABLE 3

Effect of E.S. on time to reach pH 6 (h)(1)

ES_{100} (2)		ES_{300} (2)		No ES	
Left (E.S.)	Right	Left (E.S.)	Right	Left	Right
2.18	6.23	3.24	4.99	5.00	6.00
2.42	6.00	3.92	9.13	6.20	6.38
2.79	4.84	4.44	6.99		

(1) Calculated as described under experimental.
(2) ES_{100} = lower voltage stimulation.
ES_{300} = higher voltage stimulation.

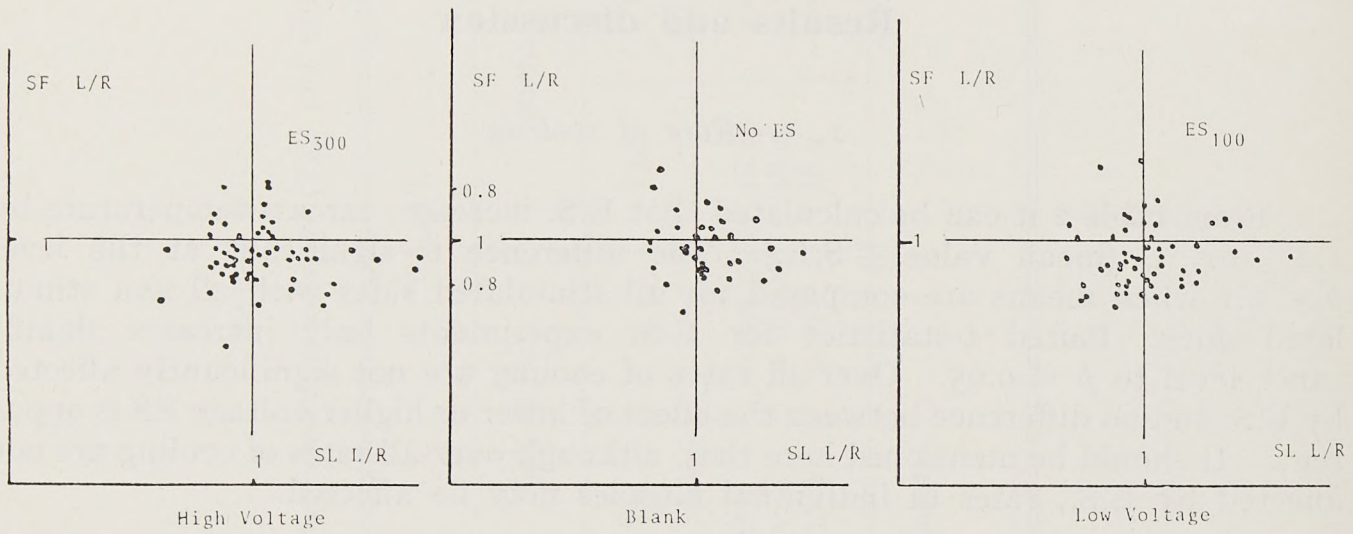

FIG. 1. — *Effect of ES on the shear force ratio left/right (SF L/R) and sarcomere length ratio (SL L/R) left/right for 20 different muscles. All muscles sampled from three (ES_{300} and ES_{100}) or two animals (no ES) are grouped.*

sarcomeres. The magnitude of tencerisation is small but evenly distributed over the various muscles as illustrated in table 4.

An overall tenderizing effect of 6 ± 2 p. 100 (mean ± S.E.) can be calculated with a tendency to be most outspoken for loin muscles and least for leg and shoulder muscles. No difference is apparent between higher and lower voltage E.S. A similar conclusion is apparent when weighed averages for shear force values over the whole carcass are calculated (table 5).

The magnitude of tenderization observed here is much smaller than that reported in other work where tenderizing effects of about 25 p. 100 were obtained (e. g. SAVELL *et al.*, 1979). In many cases however older animals and steers or cows were used showing much higher control shear force values. It would seem that the tenderizing effect obtained is dependant on the initial tenderness (see also CASTEELS *et al.*, 1979). This again suggests that tenderization is related to some effect of E.S. on connective tissue. In any event, our results indicate that

TABLE 4

Effect of E.S. on shear force ratio Left/right for different muscle groups

Muscle group (2)	No ES (2) (1)	ES_{100} (3)	ES_{300} (3)
Leg	0.98 ± 0.03	0.94 ± 0.03	0.95 ± 0.02
Neck + Breast	0.95 ± 0.04	0.92 ± 0.02	0.88 ± 0.05
Shoulder	0.99 ± 0.02	0.95 ± 0.06	0.95 ± 0.03
Loin	1.01 ± 0.07	0.92 ± 0.03	0.86 ± 0.09

(1) ( ) = number of animals used.
(2) Leg = *Tensor fasciae lat., Semitendinosus, Gastrocnemius, Biceps femoris, Gluteus medius, Vastus intermed., Semimembranosus.*
Neck + Breast = *Long. Dorsi* (3-4), *Pectoralis prof., Pectoralis superfic., Latiss. dorsi, Splenius cervicis, Serratus ventralis.*
Shoulder = *Infraspinatus, Triceps brachii, Subscapularis.*
Loin = *Long. Dorsi* (6-7), *Psoas major.*

TABLE 5

Effect of E.S. on weighed average shear force values of carcass (kg) (1)

No ES		ES_{100}		ES_{300}	
Left	Right	Left	Right	Left	Right
4.80	4.92	4.05	4.33	4.35	4.78
6.23	6.64	4.06	4.41	3.42	3.81
		4.50	4.69	4.24	4.77

(1) Calculated as described by DEMEYER *et al.* (1980).

ES tenderizes meat by a mechanism other than the prevention of cold-shortening as no differences in sarcomere length were observed between stimulated and non stimulated sides. Also, as carcasses were cooled immediately after E.S. before pH 6 was reached (table 3), both stimulated and non stimulated sides probably suffered cold shortening as discussed earlier (DEMEYER *et al.*, 1980). Tenderization may be related to an increased rate of protein fragmentation during ripening, as suggested by SDS-PAGE electrophoresis. During vacuum ageing of *long. dorsi* muscle the proportion of 30 000 D in total protein increased following $y = a + bx$ with :

$y = 2.06 + 0.21\,x$ for stimulated muscle and
$y = 2.66 + 0.14\,x$ for non stimulated muscle.

and y = % 30 000 D in total protein
x = time in days.

Paired t-statistics showed the rate coefficient b to be significantly higher for stimulated muscle ($p \leqslant 0.05$). No relationship with Troponin T disappearance could be detected however.

Wether this increased rate of protein fragmentation is related to the decreased shear force values remains to be established.

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Scénario du futur pour l'organisation de l'industrie de la viande avec la stimulation électrique et le désossage précoce des carcasses

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La troisième partie du colloque est réservée aux possibilités d'application des techniques de désossage précoce et de stimulation électrique de la musculature.

Nous entendrons des exposés consacrés à des réalisations déjà existantes et qui fournissent des éléments d'information pour une action à court terme dans la filière viande telle qu'elle existe actuellement. A côté, il a paru intéressant d'évoquer, sous forme d'une réflexion libre, quelles pourraient être les implications de l'introduction du désossage précoce et de la stimulation dans de nouvelles perspectives d'organisation de la filière et du système viande, tout spécialement dans le cas des viandes bovines.

Cette analyse est la base d'un scénario du futur qui veut, sans prétention, offrir des éléments de prospective et par conséquent de réflexion sur ce que pourrait être l'organisation de la filière, et plus précisément de de que nous appellerons le système viande.

Le système viande

Le système viande est l'ensemble de l'organisation, des opérateurs et des équipements qui permettent la transformation de l'animal en produits consommables par l'homme. Le système viande fonctionne depuis les origines de l'homme. Au départ il était inévitablement simple et supposait une utilisation ultra précoce de l'animal après la mort. Avec le temps il s'est compliqué et diversifié et il a pris des formes très variables d'un pays à l'autre. Alors même qu'il existe encore actuellement (dans des pays du tiers monde) des formes primitives du système, nous observons dans nos pays dits industrialisés des formes relativement élaborées du système. Il peut exister un grand nombre de systèmes différents qui se distinguent :

— par la nature des produits obtenus;
— par les techniques utilisées pour la transformation.

Les différents systèmes viande

La nature des produits peut être très variable et tirer un parti très différent des caractères spécifiques, particuliers, originaux des divers types de composants corporels que sont :

— les différents muscles et graisses;
— les différents éléments des viscères.

On peut envisager pratiquement tous les intermédiaires entre une utilisation indifférenciée et un emploi spécifique très diversifié.

L'utilisation indifférenciée peut s'envisager avec un minimum d'opérations technologiques ou avec une technologie très développée :

— un exemple du premier type est celui de l'emploi des carcasses dans certains pays où l'on utilise la carcasse « comme cela vient », en la débitant de la tête à la queue sous forme de tranches et de morceaux de viande fraîche indifférenciés les uns des autres. On n'ose pas parler, dans ce cas, de boucherie pour qualifier une telle exploitation de l'animal;
— un autre exemple d'utilisation indifférenciée est celui où la totalité de la carcasse est, à l'exception des os, intégralement transformée en saucisses.

Un emploi spécifique très diversifié suppose une technologie complexe et adaptée.

Dans le cas des bovins, l'utilisation qui est faite, par les professionnels français, des divers éléments de la musculature est déjà un exemple de technologie très avancée qui est un modèle de plus en plus imité dans de nombreux pays étrangers.

Caractérisation d'un système viande donné

On pourra apprécier et caractériser un système viande par différents critères et par la valeur de différents paramètres, spécialement par :

— le rendement matière, R;
— le niveau qualitatif des produits obtenus, Q;
— le coût des opérations, C.

1) Le rendement matière est important à considérer. L'animal vivant est une matière première comparable à un minerai brut. La masse des produits qu'on en extrait dépend non seulement de facteurs propres à l'animal (âge, sexe, poids, état de santé...), mais aussi des technologies utilisées. Les pertes de masse de toutes natures subies au cours des transformations sont des facteurs déterminants du rendement matière.

2) Le niveau qualitatif des produits est défini tant par leurs propriétés organoleptiques que par leur composition. On peut admettre que ce niveau détermine globalement le prix d'achat consenti par les consommateurs.

3) Il est clair que, dans notre régime économique, l'adoption d'un système de transformation particulier par une entreprise donnée est d'abord déterminée par

le montant du profit net obtenu dans la transformation. Pour une masse initiale de matière première M, ce profit peut, schématiquement, s'écrire

$$(M.R.Q) - C$$

Il est évident que le même profit peut être obtenu par différentes combinaisons du rendement matière, du niveau qualitatif des produits et du coût des opérations.

L'évolution des systèmes viande

L'amélioration des connaissances, d'une part, et l'évolution des contraintes s'exerçant dans et sur le système viande, d'autre part, sont de nature à modifier fortement le niveau du profit obtenu, en amenant à changer notamment la quantité et la nature des produits. Mais cette modification peut aussi changer la structure interne du système en modifiant les opérations unitaires impliquées dans la transformation.

Le système viande est ainsi amené à évoluer, en particulier en raison de l'évolution des contraintes qu'il subit. Dans le cas de la France, on peut admettre ainsi — et ce sera l'hypothèse de base de notre scénario — que le système viande sera appelé dans le futur à subir fortement l'influence des contraintes suivantes :

— paiement des producteurs à la qualité;
— amélioration de l'efficacité économique des entreprises;
— diminution des coûts, notamment dans le domaine de l'énergie;
— amélioration de la qualité bactériologique;
— amélioration de l'aptitude à la conservation;
— amélioration des qualités organoleptiques et garantie des niveaux de qualité offerts aux consommateurs;
— amélioration de l'utilisation de l'ensemble des composants corporels en ce qui concerne la valeur nutritionnelle, les qualités organoleptiques et la facilité d'emploi.

Le système viande du futur

On distingue classiquement jusqu'à maintenant plusieurs transformations dans l'utilisation des animaux. On parle d'une première transformation, conduisant de l'animal vivant à la carcasse, puis d'une seconde transformation amenant de la carcasse aux différentes pièces de viande, et, enfin, d'une troisième transformation aboutissant aux produits élaborés.

Compte-tenu des contraintes que nous venons d'évoquer et des perspectives offertes par la stimulation électrique des carcasses et le désossage précoce on peut supposer que les transformations successives qui sont pour le moment échelonnées dans le temps et dans l'espace seront amenées à se fondre dans un ensemble général dont la caractéristique essentielle sera de réaliser l'éclatement de l'animal en ses différents composants très peu de temps après la saignée, disons dans es deux ou trois heures.

Cet éclatement s'accompagnera de différentes opérations parallèles et, en quelque sorte, secondaires destinées à satisfaire les différents objectifs que l'on peut assigner au système par suite des contraintes évoquées plus haut.

Le système devra alors être organisé de manière à assurer le plein emploi des composants corporels, à améliorer les qualités organoleptiques, à garantir un excellent niveau bactériologique et une très bonne conservation et, enfin, à accroître son efficacité en réduisant ses coûts.

1. — *Le plein emploi des composants corporels*

Le plein emploi des composants corporels doit être envisagé sous deux aspects différents, mais non nécessairement opposés. Il s'agit d'abord d'assurer la récupération maximum des différents tissus et organes de l'animal vivant, de manière à avoir la valeur la plus élevée possible du rapport.

$$\frac{\text{poids des produits utilisables pour l'alimentation}}{\text{poids du corps de l'animal}}$$

Il faut ensuite développer une politique d'élaboration de produits nouveaux, à partir de mélanges judicieux des divers types de composants.

Ces deux objectifs imposent d'améliorer quantitativement et qualitativement — au plan bactériologique, en premier lieu — les opérations de récolte, puis celles du traitement ultérieur des différents composants du cinquième quartier :

— sang;
— viscères digestifs;
— cœur et poumons;
— graisses.

A l'utilisation directe sous leur forme brute devra nécessairement s'ajouter, pour nombre d'entre eux, des traitements de purification, d'extraction et de restructuration destinés à tirer le meilleur parti des protéines qu'ils renferment.

Au niveau de la carcasse proprement dite, les opérations de récolte (coupe, désossage, dégraissage) et de traitement (découpe en morceaux de détail, parage) devront aussi être améliorées de manière à assurer le plein emploi des composants corporels. La stimulation électrique des carcasses et leur désossage précoce permettront d'assurer, peu de temps après l'abattage, le nécessaire tri des composants en deux grandes catégories :

— celle des produits vendables comme viandes fraîches et dont la structure de la partie musculaire sera la structure naturelle de la viande en l'état;
— celle des produits destinés à des fabrications.

Cette seconde catégorie comprendra une large part des tissus gras et la fraction de la musculature dont les caractères de taille, de composition ou de texture ne permettent pas ou ne permettent plus, actuellement, le meilleur emploi sous forme de morceaux de viandes fraîches en l'état.

Les fabrications envisageables seront celles déjà connues et pratiquées (viandes hachées fraîches ou surgelées) et différentes préparations de charcuterie-salaison. Elles comprendront aussi des formes plus nouvelles de produits, comme les viandes reconstituées, reformées et restructurées obtenues à partir des portions de la musculature soumises à des traitements mécaniques appropriés destinés

d'une part à rompre leur texture naturelle et d'autre part à leur conférer une texture nouvelle proche de celle de la viande en l'état, sinon similaire à elle. Ces fabrications pourront employer aussi une partie d'autres protéines, à commencer par celles apportées par les composants du cinquième quartier.

2. — *L'amélioration des qualités organoleptiques*

Les qualités organoleptiques de la viande et des produits carnés auront dans le futur une importance accrue. La viande ne peut, en effet, maintenir la position privilégiée qu'elle occupe présentement sur le marché alimentaire que si son niveau qualitatif (au sens large, incluant non seulement les qualités organoleptiques, hygiéniques et nutritionnelles mais aussi les propriétés d'usage) surpasse ceux des produits concurrents ou imitatifs qui lui disputeront la place. Entre espèces animales une concurrence vive, déjà amorcée, opposera la volaille et les autres. La position de la viande bovine, en particulier, méritera une grande vigilance.

L'amélioration du niveau général des qualités organoleptiques, l'homogénéisation de ces qualités, la certification de la qualité (par le biais de marques commerciales, de labels, ...) sont des objectifs importants qu'il sera possible d'atteindre, dans la filière pratiquant stimulation et désossage précoce, grâce :

— au tri qualitatif des viandes;
— et à une technologie différenciée et modulée.

L'éclatement de la carcasse que réalisera le désossage à chaud permettra à la fois d'assurer un tri qualitatif des viandes efficace et l'application d'une technologie différenciée aussitôt que possible après l'entrée en rigor.

Le tri qualitatif suppose l'emploi de capteurs de qualité appropriés et devrait, en premier lieu concerner :

— le pH;
— les propriétés de texture;
— la couleur.

Il est souhaitable qu'il puisse s'appliquer à un maximum d'unités musculaires, justiciables chacune d'une technologie particulière.

L'appréciation des caractères de texture des muscles doit permettre notamment de définir le niveau de tendreté garantie et d'établir alors, sur des bases objectives, les limites de séparation entre viandes à usage de boucherie et viandes à fabrication au sens où nous les avons définies plus haut, à propos du plein emploi des composants corporels.

Le tri qualitatif effectué sur le pH et la couleur permettra de définir précisément les orientations technologiques les plus appropriées au type de viande, notamment pour la conservation à l'état frais, ou pour le type de produit à envisager dans le cas des viandes de fabrication. Le tri sur le pH doit assurer les conservations dans les conditions les plus favorables.

La technologie appropriée doit s'adapter à la fois à l'individualité de chacun des éléments de la musculature (chacun des cent muscles d'une demi-carcasse est, du point de vue technologique, un cas particulier) et aux besoins spécifiques de la distribution. Les développements envisageables concernent aussi bien les modes de conditionnement que les paramètres de conservation. Il s'agit, dans ce cas, d'assurer une maturation contrôlée et, par suite, modifiable en agissant sur le couple

temps /température. Au niveau de la technologie des actions spécifiques d'attendrissage pourraient aussi être envisagées, spécialement en vue d'améliorer la part de la tendreté due à la composante myofibrillaire des muscles qui resterait éventuellement à modifier après les effets de la stimulation et de la maturation contrôlée. Un attendrissage intéressant la composante conjonctive peut aussi être envisagé pour certains types de muscles.

Le désossage précoce et le tri qualitatif des viandes fourniront rapidement après l'abattage tous les éléments nécessaires pour permettre un paiement des carcasses selon leur valeur d'usage et donc selon leur qualité réelle. Celle-ci s'établira à partir du bilan matière qu'on dressera facilement en pesant la masse de muscles et de graisses utilisables et la masse des os. Le rendement en viandes utilisables sera connu précocement et pourra intervenir comme l'un des éléments déterminants du classement des carcasses, l'autre élément étant fourni par le niveau qualitatif des muscles résultant de leur texture, de leur pH, de leur couleur, etc... Le paiement à la qualité réelle des producteurs est un moyen important, et peut être le seul concrètement envisageable, d'associer ces derniers à l'œuvre qu'il est nécessaire d'entreprendre à tous les stades de la filière pour améliorer la qualité des animaux.

3. — *L'amélioration de la qualité microbiologique des produits*

L'amélioration de la qualité microbiologique — et en premier lieu de la qualité bactériologique des produits — est toujours une nécessité pour obtenir un produit de bonne conservation et pour fournir aux consommateurs un produit sain.

Les perspectives offertes à la future technologie du système viande utilisant stimulation électrique et désossage à chaud vont renforcer — et non diminuer — l'importance et, par conséquent, la nécessité de cette amélioration.

Les opérations à réaliser pour assurer le plein emploi des composants et pour améliorer les qualités organoleptiques ainsi que celles destinées à accroître l'efficacité du système supposent en effet des schémas de travail *momentané* à des températures qui sont situées en dehors des zones recommandées par les hygiénistes pour assurer — par non multiplication des germes — la stabilisation des denrées. Le problème se pose en particulier pour le nécessaire palier de température après stimulation tant que le pH n'a pas atteint la valeur minimum souhaitable pour éviter le cryochoc. Le problème se posera aussi quand on voudra pratiquer une maturation accélérée. Même si les durées de séjour à ces températures sont limitées et même si le niveau de température est relativement peu élevé (par rapport à la zone de température optimum pour la multiplication des germes à éviter) il n'en demeure pas moins que le risque d'une dégradation de la qualité hygiénique pourra subsister. Il conviendra dès lors de le limiter au minimum en agissant :

a) sur les facteurs qui déterminent les apports microbiens et qui sont donc à la source de la contamination des produits.

b) sur ceux qui peuvent modifier les caractéristiques physico-chimiques des viandes dans un sens favorable à une surmultiplication des germes ou du moins à celle de certains d'entre eux.

* *La limitation des apports* passe par :

— l'amélioration de la propreté corporelle des animaux et donc par leur nettoyage efficace avant l'entrée dans l'abattoir;

— la révision des installations d'abattage, impliquant la séparation physique des différents segments de la chaîne d'abattage et une régulation des mouvements de fluides (eau et air);
— la révision des techniques de dépouille;
— le recours à des procédés de décontamination par voie physique ou chimique.

** *Les facteurs qui sont susceptibles d'intervenir sur la croissance des bactéries* sont à contrôler par le biais :

— de l'amélioration des conditions de transport et de stockage des animaux qui influent sur le niveau du pH *post-mortem*;
— des atmosphères de conditionnement qu'il y aura lieu de contrôler soigneusement et de définir, précisément, en fonction des objectifs et des délais imposés pour la conservation.

4. — *L'accroissement de l'efficacité du système et la réduction de ses coûts*

En elle-même, comme cela sera expliqué par d'autres conférenciers, la mise en pratique du désossage précoce va conduire nécessairement à une amélioration de l'efficacité du système viande et à une réduction de ses coûts, en raison principalement de la diminution des coûts de réfrigération (équipement et fonctionnement). Le plein emploi des composants corporels est aussi, en soi, un facteur important de l'accroissement de l'efficacité du système.

Celle-ci peut aussi être améliorée en tirant le meilleur parti possible des caractéristiques de la viande chaude (en fait du muscle pantelant) en ce qui concerne les caractères technologiques (pouvoir de rétention plus élevé, propriétés émulsifiantes, pénétration de la saumure), et la température. Le choix des types de fabrication à envisager devra être soigneusement élaboré en conséquence.

L'optimisation des transformations, pour la réalisation des produits fabriqués, impliquera pratiquement une *technologie très avancée* pour la stimulation de manière à ce que dans une carcasse donnée on puisse à la fois tirer parti des avantages de la stimulation électrique (qui va faire diminuer rapidement le pH) et de ceux d'un désossage précoce de viandes pour transformation dont on souhaiterait que le pH fût le plus stable et le plus élevé possible.

Conclusion

Telles sont les grandes lignes du scénario.

Un scénario n'est pas un plan et ne dit rien de détaillé sur l'ensemble des équipements et des techniques nécessaires à son exécution. Comme dans les films cela est l'affaire du réalisateur. Ce que l'on doit indiquer cependant c'est que la faisabilité des différences opérations impliquées dans ce scénario paraît possible à très court terme ou même immédiatement dans de nombreux cas, mais pour d'autres opérations nous sommes encore, pour un temps, limités par nos méthodes et nos appareils. On peut raisonnablement penser que dans le futur, qui est l'échéance de nos préoccupations, ces facteurs limitants auront totalement disparu.

Les conséquences des changements auxquels nous venons de faire allusion

seront considérables. Nous ne les évoquerons pas ici car elles font partie du programme de cet après-midi. Mais ce que nous pouvons dire, globalement, c'est que l'exécution du scénario va mettre en œuvre, de façon conjointe et simultanée, un ensemble de techniques et de procédés dont la conception, la mise en place, la gestion quotidienne, le perfectionnement demanderont aux opérateurs des connaissances et des compétences sans cesse accrues. Et cela sera sans doute la révolution, annoncée dans le titre de ce colloque, à laquelle il faut s'attendre dans la filière viande du futur qui sera, pour cette raison, raccourcie dans le temps et plus concentrée dans l'espace.

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Electrical Stimulation and Hot Processing British Experience

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Introduction

The Meat and Livestock Commission (MLC) is a development organisation, established by Act of Parliament in 1967, to promote improvements in the efficiency of meat production and marketing. It is concerned, therefore, to achieve improvements from the conception of an animal through to its consumption. Its role is somewhat similar to the combined functions of the French organisations, ONIBEV, ITP, ITEB, ITOVIC and ADIV. MLC's development programme is financed by a statutory levy on the industry, but services provided for the benefit of individual companies are normally on a fee-paying basis.

Like their colleagues in other countries, British abattoir operators wish to improve their competitive position by making improvements in the efficiency of their operations. The two developments being considered in the present colloquium — electrical stimulation and hot processing — alone or in combination offer the potential for them to achieve this. However, commercial uptake of such new technology is slow as meat traders need convincing of the financial benefits of change, particularly one involving such a fundamental change of operation as hot processing. MLC's task is to help traders to have more confidence in taking such commercial decisions.

Electrical Stimulation (ES)

Some abattoirs, especially those wishing to export sides or quarters of beef want to chill rapidly to achieve 7 °C in the deep leg by 24 h after slaughter to allow quicker turnover and reduce chilling space requirements. Such rapid chilling also reduces weight loss to such an extent that if one measures the loss in carcases chilled to 7 °C in 24 h compared with 48 h, the saving is about one percentage unit of carcase weight. Similar advantages of rapid cooling apply to lamb.

ES allows abattoirs to achieve the benefits of rapid cooling without any dete-

rioration in quality as a result of cold shortening. But, as shown in Table 1, there is an advantage in applying ES even under slower cooling conditions. This has been demonstrated by GEORGE, BENDALL and JONES (1980), who measured the texture of meat from stimulated and unstimulated beef carcases held at 16 °C for 8 h after death and then at 1 °C for up to 21 days. Meat from the stimulated carcases was more tender at 1 day *post mortem* than that from the unstimulated controls and, although the difference was less marked at 21 days, the ES treated meat remained more tender. Recent joint Meat Research Institute/MLC work in commercial plants confirms that ES hastens the onset of ageing in at least some muscles under cooling conditions which avoid cold shortening. However, there may be little difference in texture after several weeks ageing.

Encouraged by the potential benefits of ES, MEDAL (Meat Equipment Development Association Limited), a consortium of five British abattoir equipment manufacturers, developed an ES unit which has been operating successfully in a British abattoir since late 1979 and is the first on-line automatic ES unit in Europe. The unit was designed after research to find the conditions best suited to achieve the rapid onset of *rigor mortis* throughout all of the muscles of the

TABLE 1

Benefits of Electrical Stimulation under two contrasting cooling regimes

Cooling rate	Evaporation weight loss	With ES	Without ES
Rapid cooling less than 24 hours	Low delete dots	No cold shortening Some acceleration of ageing	Cold shortening No acceleration of ageing
Normal cooling 36-48 hours	High and centre words	No cold shortening More acceleration of ageing	Probably no cold shortening Normal slow ageing

carcase. It was found that the maximum effect of ES was obtained by stimulating with a high voltage (700-1 000 v) along the entire length of the carcase as soon as possible after death. In the abattoir chosen for the first installation the best site for the unit was on the dressing line after evisceration and before carcase splitting, but the design can be applied to other positions on the line.

A key consideration in the design was that it should be able to operate with complete safety for personnel, particularly under the adverse working conditions of an abattoir, and with reliability which is ensured by the careful selection of materials and the rugged construction of the unit.

To achieve good electrical contact and stimulation along the length of the carcase, the current is passed from the severed neck muscles to the Achilles tendon. The lower electrode is a stainless steel pan shaped to contain the severed neck of the carcase and is mounted in such a way that good electrical contact is maintained throughout stimulation, even when carcases varying greatly in size are presented for automatic stimulation. The upper electrode takes the form of the 2 hooks through the Achilles tendon making contact with the earthed rail. Figure 1 illustrates the pan electrode arrangement within the safety cabinet.

The voltage applied across the carcase consists of mains derived discrete monopolar sinusoidal pulses. The peak voltage is adjustable from 250-1 000 v and the pulse frequency is adjustable from 16.7 to 50 pps. A fuller description of the unit, its safety features to meet the high standards of Britain's Health and Safety Executive, and its operation has been given by HOLT, CUTHBERTSON and GIGIEL (1980).

Results obtained from using the MEDAL unit demonstrate that it is achieving the objective of reducing muscle pH rapidly after death. Table 2 illustrates some of these findings.

Preliminary estimates of the cost effectiveness of ES indicate a significant financial advantage. A differential in favour of ES of 0.22 FF /kg saleable meat

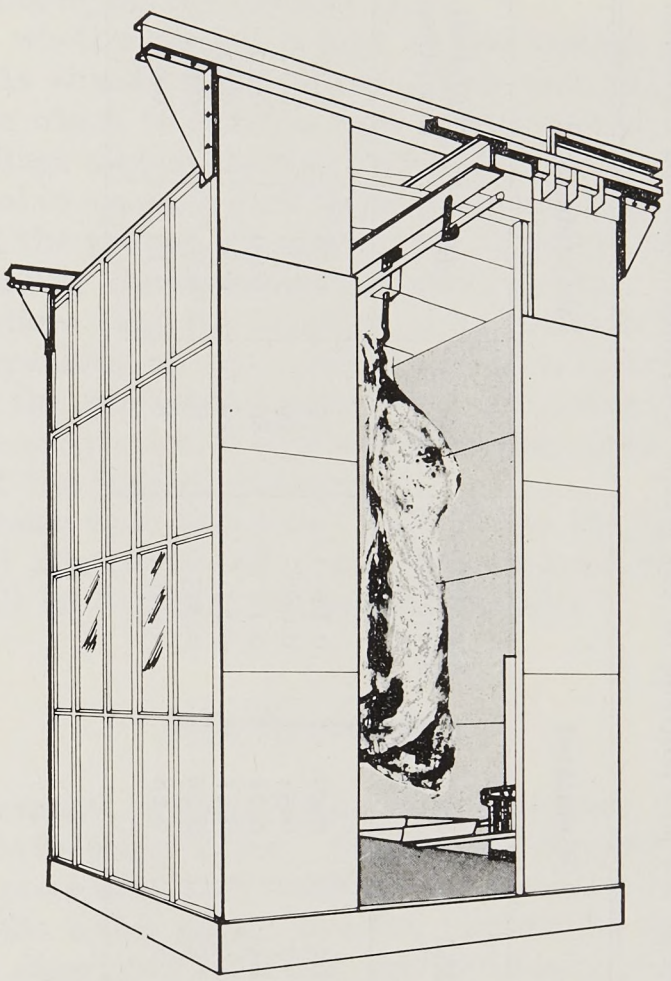

FIG. 1. — *Illustration of MEDAL's automatic ES unit.*

has been estimated for an abattoir applying ES compared with a similar abattoir without this facility. While this may seem a small amount for an individual carcase it amounts to some 40-50 000 FF per week for an abattoir handling 1 000 cattle per week. In making the estimates it has been assumed that the abattoir applying ES is chilling to achieve 7 °C in the deep leg in 24 h followed immediately by boning and vacuum packing, with ageing in packs for 8 days before despatch. The other abattoir is assumed to chill to 7 °C over 48 h, followed by boning and ageing of the meat in packs for 8 days. Most of the financial advantage stems from the saving in weight loss noted earlier in this paper. No allowance has been made for any quality advantage which may accrue from ES, such as improved colour or texture.

TABLE 2

Results obtained from using MEDAL unit in a commercial abattoir (Unit set at 700v, 25 pps for 90 seconds) *Muscle pH (approx. 1 h* post mortem) *in four groups of stimulated and non-stimulated carcases*

Group	Muscle	Stimulated			Non-stimulated			Difference	Standard error of difference
		No.	pH	Standard deviation	No.	pH	Standard deviation		
1 Steers	SM	65	6.49	0.14	77	6.76	0.11	0.27	0.022
1 Steers	PS	67	6.54	0.080	79	7.02	0.12	0.48	0.017
2 Steers	LD	105	6.21	0.22	86	6.60	0.20	0.39	0.030
3 Cows	LD	19	6.21	0.17	19	6.62	0.22	0.41	0.066
4 Steers	SM	8	6.30	0.093	8	6.62	0.082	0.32	0.047
4 Steers	TB	8	6.28	0.041	8	6.76	0.056	0.48	0.026

SM — M. semimembranosus.
PS — M. pectoralis superficialis.
LD — M. longissimus dorsi.
TB — M. triceps brachii.

From: HOLT, CUTHBERTSON and GIGIEL (1980).

In recent months the British industry has shown interest in low voltage ES equipment, partly because its cost is much less on the assumption that it will not require the same safety protection for personnel as high voltage equipment, and partly because the shortage of space on the dressing lines in some plants makes it difficult to install high voltage equipment with the necessary safety cabinet. MLC has been fortunate to be able to conduct some test work with the Swedish MITAB equipment which involves stimulation through the nose or ear within the first few minutes of stunning. The results so far indicate that, given good operating conditions, low voltage ES can produce as fast a reduction in pH as high voltage stimulation applied about 30 minutes *post mortem*. To achieve a good response with low voltage ES it seems vital to stimulate within 5 minutes of stunning and to have good electrical contact. In practice this seems to require clean shackles and hooks linking to the earthed rail.

The decision on whether to use a high or low voltage system will depend on several factors. As already noted, these include cost and the space available on the dressing line in which to install a high voltage system. In the absence of an automatic low voltage system, further factors to be considered are the extent of management control in ensuring staff apply the low voltage system consistently to every carcase, and the rate of kill which, with high throughput, could put a strain on existing staff in the stunning and bleeding area.

There are indications that the duration of low voltage ES may have to be set for each plant depending on the general level of non-carcase electrical resistance encountered in the stimulation system and the extent to which stiffness in the carcase, due to the early onset of *rigor mortis*, creates carcase dressing problems for staff especially at the legging stations.

With more evidence now becoming available on the benefits of applying ES and the merits of alternative ES units, it is expected that the commercial application of ES will be extended in British abattoirs.

Hot Processing

The scientific literature contains many reports of work indicating the benefits of hot processing in its many forms. A paper by CUTHBERTSON (1980) reviews the rationale of hot processing and considers its economic implications.

Despite the benefits which appear to exist, commercial uptake is slow, particularly to the degree illustrated for a hot beef processing unit in Figure 2. At present in Britain there is only one plant carrying out hot processing and, at this stage, it consists largely of deboning and vacuum packing the hindquarter cuts. There are several reasons why commercial uptake has not been faster, and an important one is that the shape of the hot boned vac-packed primal cuts tend to look a different shape from cold boned vac-packed primals causing resistance from retailers.

However, MLC is encouraged by the laboratory scale trials and some commercially based test work in other countries, and is keen to see hot processing technology applied more widely in Britain. In order to provide industry with the information it needs about the advantages and disadvantages of applying such procedures commercially, MLC, in co-operation with the Meat Research Institute and some commercial meat firms, has started a development programme.

From the work in Britain and elsewhere it seems that the weight of meat going into the vacuum pack is very sensitive to the degree of fat and bone trim-

ming. It is easy to cut off either too much or too little fat from the hot carcase, and bones from hot carcases tend to be heavier due to more meat being left on them. There are indications that, even if the level of fat trim were the same hot and cold, the weight of meat going into the vac-pack would be little different. This appears to arise because, compared with the cold boned carcase, the saving in weight in cooling is balanced by the greater weight of bone. However, the

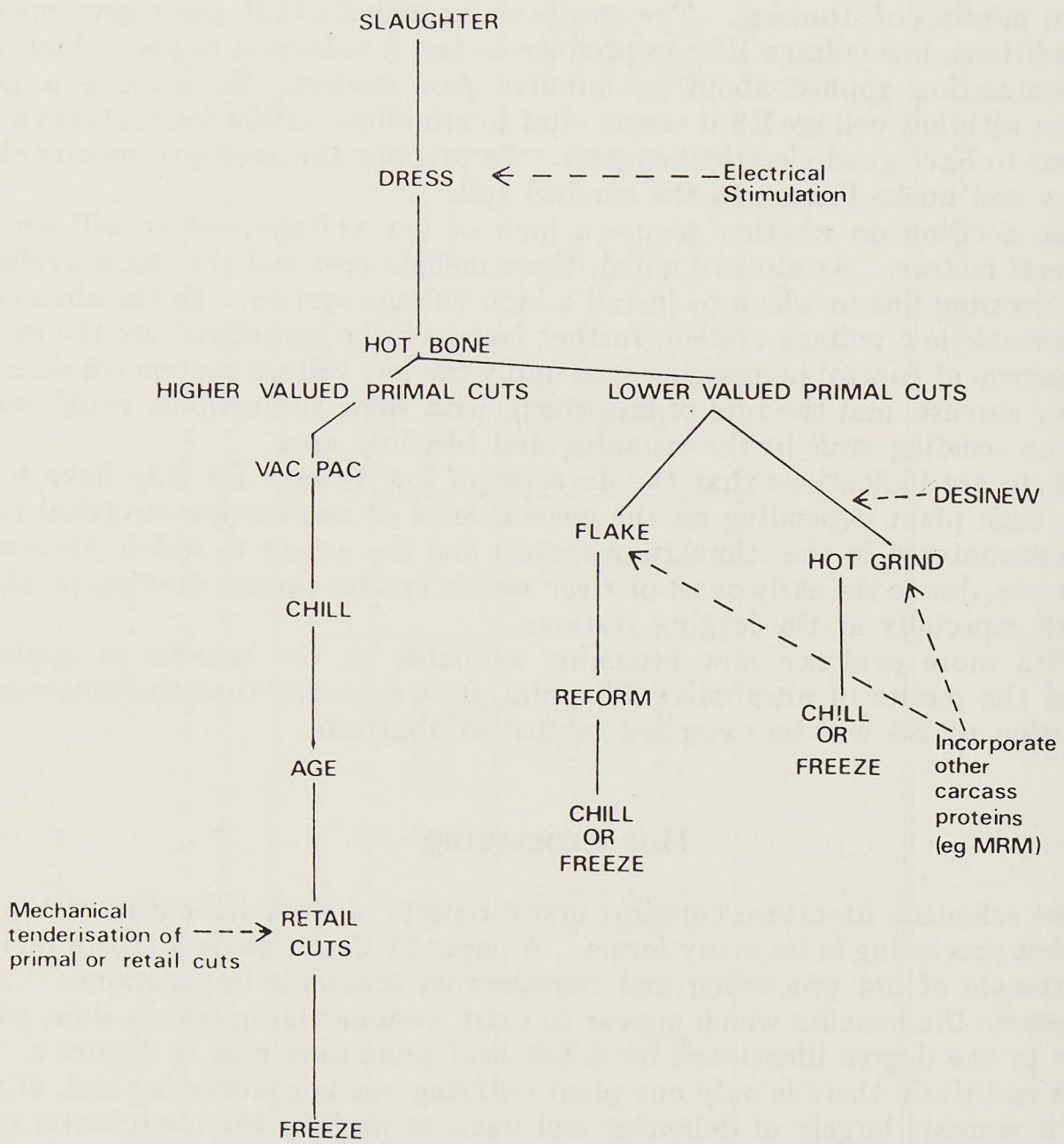

FIG. 2. — *Flow chart for a possible hot beef processing unit (the dotted lines indicate additional processes) which could produce improvements in quality and use of resources.*

extra meat left on the hot bones could be recovered in a mechanical recovery system. Table 3 gives the results of some primal cutting by TAYLOR, SHAW and MACDOUGALL (1981), and in this case shows that the advantage in meat yield is derived largely from the lower fat trim on the hot carcases.

At the stage of retail preparation, a higher yield of prepared retail cuts is likely to be achieved from hot boned primals, even if the weight of primal meat going into the vac-pack is the same both hot and cold. This is largely because there is less waste trim due to the better condition and colour of the cuts. So far

as the shape of the cuts is concerned, it appears that while the primal cuts may look a different shape, partly because of differences in cutting techniques applied to hot and cold carcases, the shape of the prepared retail cuts is likely to show very little difference.

Concern is often expressed about the potential bacteriological problems associated with hot processing under commercial conditions. The evidence suggests that satisfactory quality can be achieved in this respect provided the necessary steps are taken to have a hygienic operation and that the meat is cooled as quickly as possible after slaughter. Where hot cut primals are being cooled in

TABLE 3

Yield of saleable meat, fat trim and bone from hot and cold boned sides of beef

(Results from sixteen carcases where one side was hot boned and vacuum packed and the other side was cold boned after 48 h and then vacuum packed)

	% of hot side weight	
	Hot boned	Cold Boned
Cold side, 48 h after slaughter		98.3
Saleable meat from forequarter	38.1	36.0
Saleable meat from hindquarter	38.1	37.6
Total saleable meat (including lean trim and kidney)	76.2	73.6
Fat Trim	5.6	8.2
Bone	17.6	16.3
Evaporation loss during jointing, boning and packing	0.6	0.2
Evaporation loss during chilling	0	1.7
TOTAL	100.0	100.0

From: TAYLOR, SHAW and MACDOUGALL (1981).

vacuum packs mounted on racks, it seems desirable to have a horizontal air flow across the packs to achieve an even cooling rate. The application of ES to carcases which are to be hot boned, facilitates the rapid cooling of hot cuts without the danger of cold shortening.

Turning to the financial benefits of hot processing, MLC has estimated that even if there were no yield advantage from hot boning at the primal stage, an abattoir built to slaughter, hot bone and vacuum pack primal cuts compared with one operating a conventional cold boning and vacuum packing system, could show an improvement in return on invested capital of 7 percentage units. This arises partly from the estimated saving of 10 per cent in the capital cost of the building and partly from the saving in labour. If there were also a yield advantage at the primal stage this would improve the return on invested capital substantially. If one follows through the range of procedures outlined in Figure 2, then the increase in the value of the product for sale could be of the order of 18 per cent.

The application of hot processing technology, with or without the use of electrical stimulation, offers the potential for the British industry to improve its efficiency considerably. At the same time it provides the opportunity to create an interesting range of meat products to satisfy the growing demand for convenience foods.

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Industrial application of low voltage electrical stimulation in Sweden

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All slaughterhouses in Sweden owned by Scan — The Farmers Cooperative — are now using low voltage electrical stimulation (MITAB, Simrishamn, Sweden). This means that about 80 per cent of the beef meat produced in Sweden is electrically stimulated. Under prevailing Swedish conditions using lean animals and fast chilling, a substantial improvement in meat quality has thus been achieved. This paper will give some ideas about the practical experiences obtained.

Stimulation is generally performed immediately after debleeding, on the bleeding rail, by manually attaching a contact clip to the nose of the animal. The carcass is earthed through the shackled back leg and the rail. Electrical conditions are as given by RUDÉRUS and FABIANSSON (1980). Stimulation time is usually 1 min. The system is used on slaughter lines having a capacity of up to 45 animals per hour.

Figures 1 and 2 give typical examples of the kind of pH curves that are obtained in stimulated and nonstimulated young bulls slaughtered under commercial conditions (FJELKNER-MODIG *et al.* to be published). Stimulation is seen to have a profound effect producing much faster pH fall and also more reproducible individual pH values than in nonstimulated controls. Fast cooling can be started within 2 hours without risk of cold-shortening.

In order to establish a simple quality control the following control program has been recommended by the Swedish Meat Research Institute. The pH in M. *longissimus dorsi* of each animal is measured about 1 hour after stimulation. If the pH is 6.5 or below the carcass is passed. If the pH is above 6.5, pH is again measured after 24 hours. If this second pH value is 6.2 or above the carcass is labeled as DFD or dark cutting and the meat should not be vacuum packed. If the pH is below 6.2 the carcass is considered normal but not sufficiently stimulated and the meat should not be quaranteed for tenderness.

The pH limits presently used may be lowered in the future when more experience has been gained about the precision of routine pH measurement and the long term reliability of the stimulation process.

The possibility of discerning between normal and DFD carcasses already

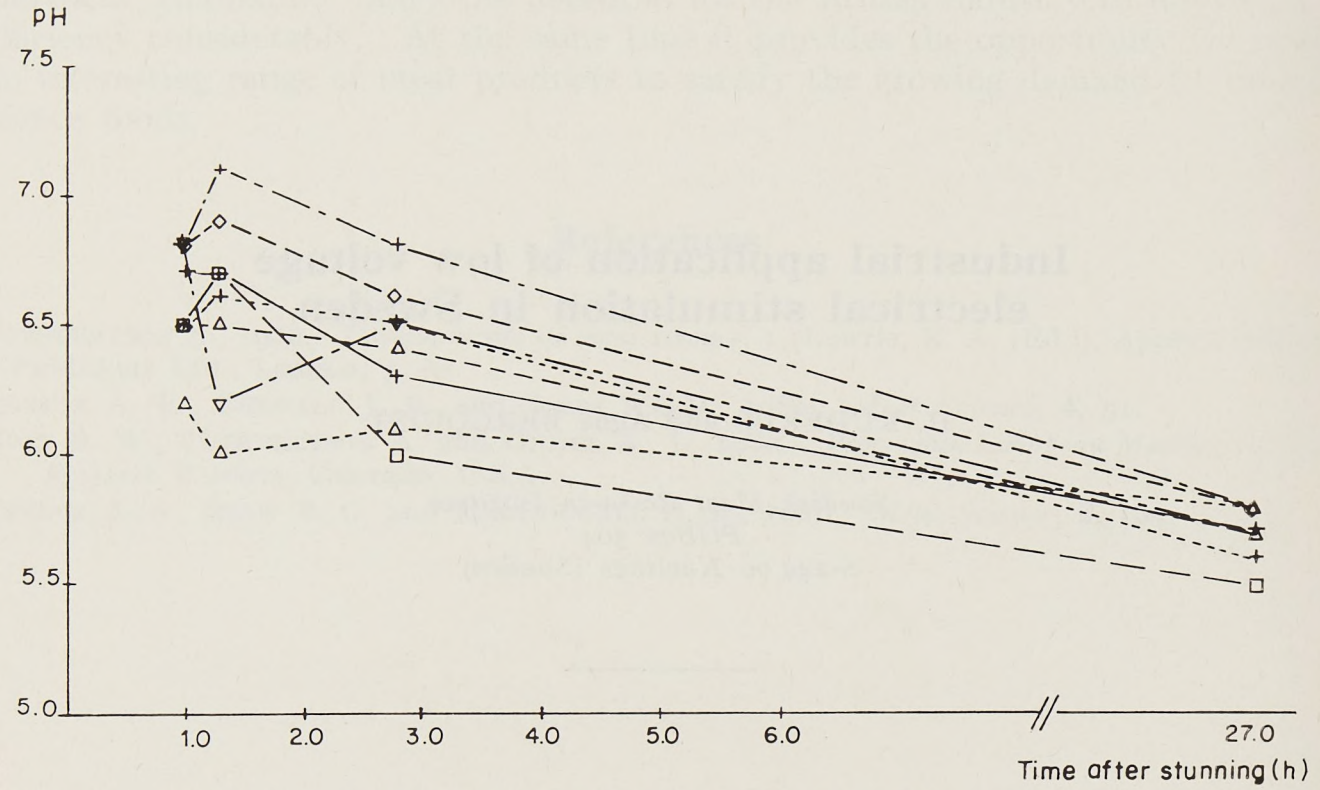

FIG. 1. — *pH values in M. longissimus dorsi from 8 young bulls. Measurements of pH were made 1.0, 1.3, 2.8 and 27 hours respectively after stunning.*

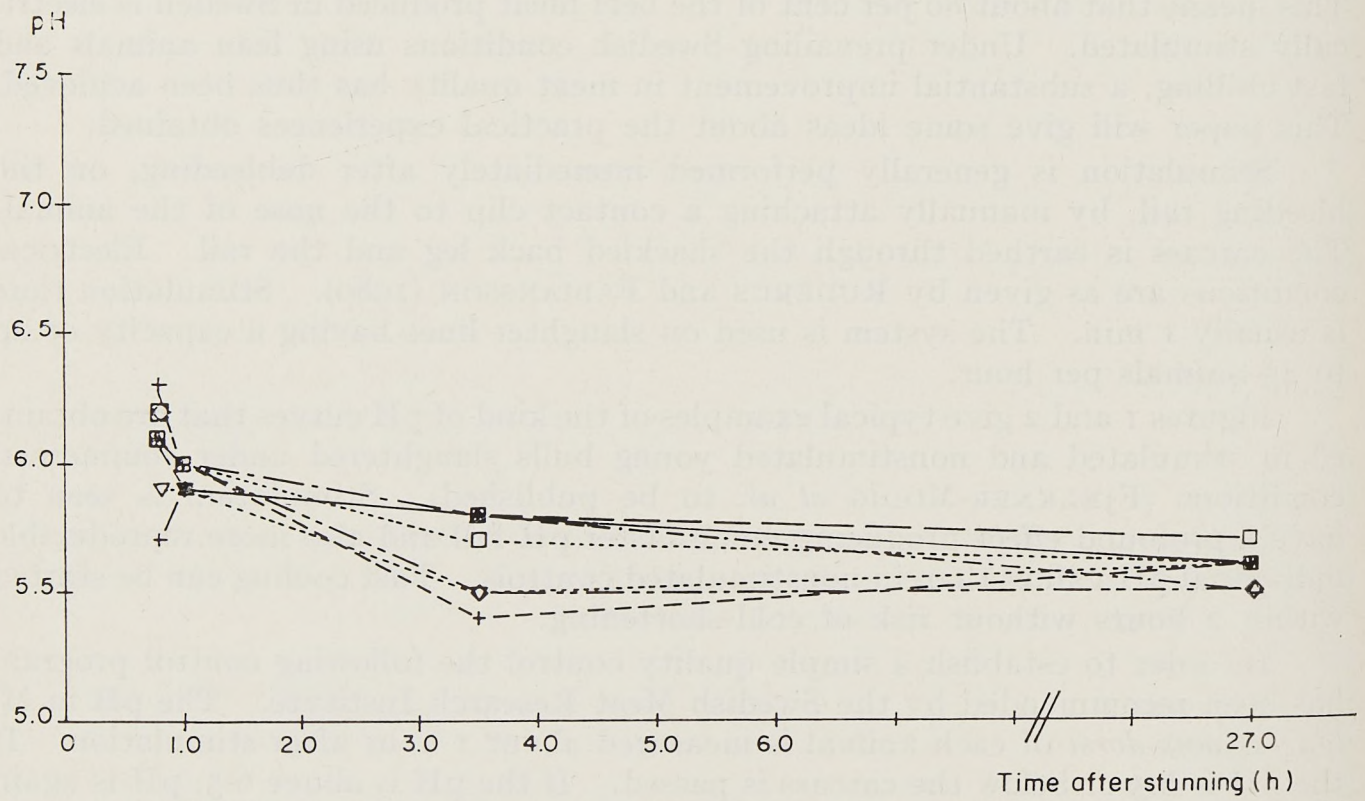

FIG. 2. — *pH values in M. longissimus dorsi from 8 young bulls. The carcasses were electrically stimulated (85 V, 32 s) immediately after exsanguination. Measurements of pH were made 0.8, 1.0, 3.5 and 27 hours respectively after stunning.*

at the first pH measurement has been considered. If the stimulation is working properly and the pH limit is slightly lowered it should be possible to use this single pH measurement as a means of distinguishing between dark cutters on one hand and normal plus border-line carcasses on the other.

Problems of insufficient stimulation have been encountered in some places. These have always been caused by bad electrical contact between back leg and conveyor rail due to rust, grease, etc.

In some cases there have been complaints from the workers about stiff carcasses during dressing. This is thought to be due to early rigor caused by too efficient stimulation. In extreme cases this has been remedied by decreasing the stimulation time.

Electrically stimulated meat is now marketed in Sweden by Scan with an unconditional tenderness guarantee. This is marked by a special label (fig. 3). When retailers buy stimulated primals they also get extra labels which they can put on their consumer packages in the shop. If the consumer is not satisfied with the tenderness of the meat he can present the label from the package and some

FIG. 3. — *Label for tenderness guarantee used for electrically stimulated meat in Sweden.*

of the meat and get his money back. The cuts that are presently guaranteed are cuts from the loin and the hind part being used for steaks and grills. This represents about 16 per cent of the dressed carcass.

The advantages of low voltage electrical stimulation noted by the Swedish meat industry are listed in table 1. That no aging is needed is explained by the

TABLE 1

Advantages of low voltage electrical stimulation as used in Sweden

No safety precautions.
Simple equipment.
No extra space needed.
More efficient debleeding.
Very fast pH-fall in all muscles.
No difference between back legs.
Conditioning on slaughterline.
Fast chilling after 2 hours.
No aging needed.
Increased overall quality in marketed meat.
Tenderness can be guaranteed.

fact that the normal handling and distribution time for beef meat is about one week before the meat reaches the consumer. This is considered enough to give acceptable tenderness.

No disadvantages have been noted so far. The increased drip indicated in laboratory experiments has not been of practical importance and is probably counteracted by the shorter aging time.

Low voltage stimulation is thus industrially established in Sweden and is generally considered to benefit both industry and consumers.

Acknowledgement

We thank Stina FJELKNER-MODIG for permission to use the pH curves shown in figures 1 and 2.

Reference

RUDÉRUS H., FABIANSSON S., 1980. Research on low voltage electrical stimulation of beef carcasses in Sweden. *Ann. Technol. agric.*, **29**, 581.

Ann. Technol. agric., 1980, **29** (4), 663-665.

Hot boned beef

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Perhaps it would be reasonable to give a short description of what Celebrity stands for.

After an amalgamation of 5 different slaughterhouses in 1970 the administration was concentrated at the head office in Esbjerg, which was established already in 1888.

Celebrity is a co-operative association which is owned by approx. 7 000 members. The total number of employees is about 1 000 persons. The turnover for the present financial year will be about 950 million Danish Kroner.

In Esbjerg the annual slaughter of pigs amounts to 500 000 and besides, there is a considerable production of canned meats, of which a great part is exported to many parts of the world.

To a great extent our sales take place through agents with the exception of United Kingdom, where we have our own sales office.

On a yearly basis Celebrity slaughter 90-100 000 head of cattle, of which approx. 50 p. 100 are young bulls which are exported as carcasses to Italy.

The remainder are cows, which are mostly slaughtered at our Rodding plant, which is situated in the southern part of Jutland. My paper is going to deal with this factory.

The Rodding plant employs 180 people and it is here that we have specialized in « hot boned beef ».

For a number of years Celebrity has gathered experience in connection with vacuum-packing beef, as we have produced vacuum-packed chilled meat since 1966.

The development of the « hot boned » beef started as an experimental arrangement in 1976, when Celebrity carried out a series of experiments on its own.

At an early stage it became obvious that this project had a future, but at the same time it was clear, that we would face some problems production-wise which had to be solved. Therefore, around the middle of 1976 The Danish Meat Research Institute was involved in the experiments, so that we could clarify as soon as possible, whether the project should be put into production.

At that time our Rodding factory was not yet ready so that the experiments

could not be made there. Therefore the work, which consisted of 3 main experiments, was carried out at our branch in Tonder.

One of our main problems was to avoid cold shortening, and therefore we operated with different chilling temperatures and chilling times in connection with the experiments. These experiments were carried out with special permission from the Veterinary Authorities in Copenhagen.

As we felt that our experiments production-wise by now were so positive, the company's board of directors, as well as the management decided that the project was to be put into an organised production, and the rebuilding of the factory was started. By the month of april 1978 the conversion was finished. Our aim was to reach a capacity of approximately 500 animals pr. week during the introduction period and now the slaughter has reached a level of 8-900 animals pr. week. So, on the whole we feel that we can be satisfied both with the decision which was taken, as well as the development that followed.

In order to give a short resumé of the whole slaughtering process we can say that about 80 p. 100 of the animals are supplied directly by the farmers, whereas the rest is bought on markets.

The slaughter of the animals is carried out in a hanging position, and afterwards the carcasses are cut into forequarters and hindquarters. The hindquarters are deboned immediately after slaughtering and approx. 30 p. 100 of the forequarters are also hot-boned.

It is obvious that if we are talking about hot-boned beef, it is essential to carry out different control measures, as meat which is vacuum-packed at a temperature of 35-37 °C offers ample possibility for growth of bacteria if very rigid hygienic demands are not met. This is the reason why it is necessary to keep personnel who are working with the slaughtering-process separated from those who are occupied with the hot-boning, and staff are not permitted to walk around freely outside their own section.

In order to maintain this separation workers on the slaughter line wear red caps, whereas the people occupied with the hot-boning wear blue caps.

One of the things which caused trouble was the actual boning technique, but after a few experiments we found out that the right way to do this was by boning on the rail. We have the impression that personnel are happy with this working process.

Moreover it is essential to keep a high hygienic standard and all gloves and knives are thoroughly cleaned after each interval. Four times a day the workers change their aprons and cutting boards are replaced. The containers transporting the meat are cleaned after each transport.

With regard to the deboning itself we try to keep the single muscles as intact as possible, because we think, that this gives a better shelf-life and a lower drip loss.

The hot-boned cuts are vacuum-packed and chilled, first at a temperature of 12 °C for a period of 5 hours, and then at a temperature of 7 °C for the rest of the day. The following day the packages are brought to the ripening room, where the temperature is 2 °C.

After 2 days of chilling the vacuum-packed cuts are placed in cartons, which are kept in our warehouse until delivery, also at a temperature of 2 °C.

The reason why we do not pack the finished product into cartons immediately is due to the fact that we wish to make sure that leakers are not going to be delivered.

Trimmings are mainly used in our canned meat production, and the final trimming of the bones is carried out with a bone-trimmer here.

We feel ourselves that we have gained considerable advantages through this method of production, especially with regard to production technique. Finally we believe that the hot-boning method itself together with the new packing technique has contributed to our achieving the manufacture of a product which in a very short time has been accepted by our customers.

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Typical italian abattoir Cuts there obtained boning while warm Cutting procedures

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It is necessary to provide some introduction facts before proceeding to the description of a typical Italian abattoir and the cuts there obtained.

The beasts have an average live weight of 165 kg yielding a carcase of 65 kg, and are 11 months old. Genetic research, feeding and age all reflect the fact that, from an economic point of view also, the most important items for the Italian processing industry are raw ham, « salami » and « coppa », the production of which is based above all on the maturation obtained by salting and dehydration.

In slaughtering the highest level of productivity in terms of beasts per hour and highest quality meat from a technical and bacteriological point of view are obtained by the complete preparation while warm of all the cuts to be used boned in processing, and the consequent rapid chilling.

It is not normally possible to achieve this, above all for economic reasons: this system requires very high manning levels on the production line at certain times, with other periods in which the workforce is idle.

In general, therefore, compromise solutions are adopted to obtain the best technical and economic results.

It can thus be seen that in Italy the capacity of an abattoir depends on 4 factors: 1) the speed of the production line, 2) manning levels, 3) the capacity of the chilling rooms, 4) the rate of turnover of the meat in the chilling rooms.

Referring to the slaughtering and cutting process for all the typical Italian cuts and to the refrigeration capacity for their complete chilling to 0/+ 2 °C, we will see that the production capacity of a plant depends to a large extent on the capacity of its chilling rooms expressed in m^2 of surface area, because of the way in which the meat is stored.

Description of a typical abattoir — Means of transport

The abattoir which we shall examine can be considered capable of dealing with 400 beasts per day.

The slaughtering is carried out in an area separate from the area where the carcases are cut.

Technical features (fig. 1) :

1) Waiting area (20 × 35) = 700 m²
2) Slaughtering area (20 × 35) = 700 m²
3) Cutting area (40 × 25) = 1,125 m² (air conditioned
4) Chilling ante-room (45 × 3) = 135 m²
5) Chilling rooms :

A (10 × 7) = 70 m²
B (10 × 7) = 70 m²
C (12 × 7) = 84 m²
D (4 × 7) = 28 m²
E (10 × 10) = 100 m²
F (4 × 13) = 52 m²
G (4 × 10) = 40 m²

TOTAL = 444 m²

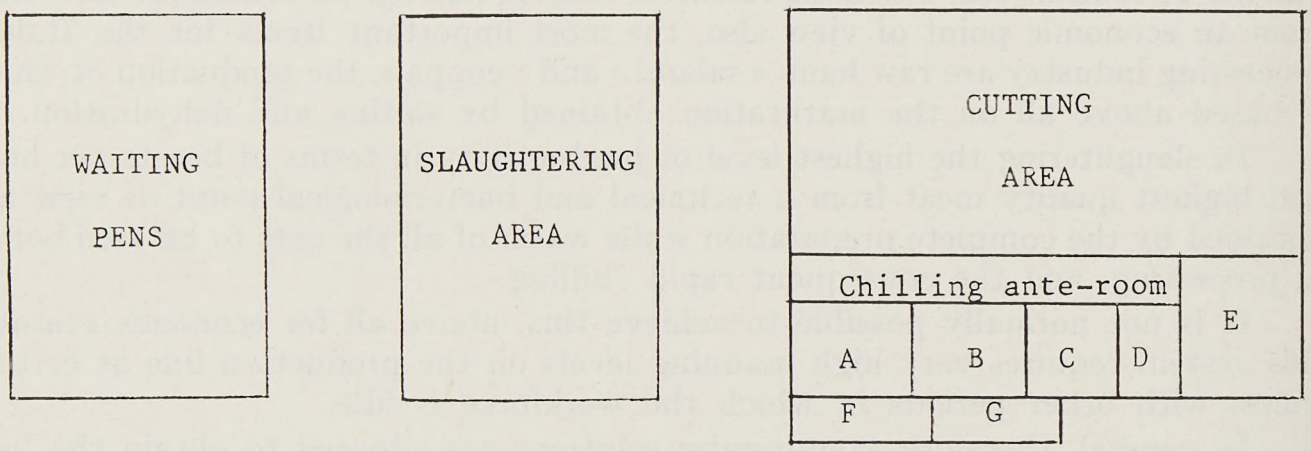

FIG. 1.

The cutting area has a motorized overhead conveyor for the transportation of the carcases.

Down its long side is the cutting bench: length 3.5 m;
breadth 1.6 m;

with 4 lateral benches: length 5.5 m;
breadth 1.6 m;

The bench has a central conveyor belt of 40 cm, and fixed sides which can be inclined.

The chilling rooms are powered by ammonia with the expansion coil at — 10 °C.

They are ventilated at an air outlet speed of 0,20 m/s.

Now the paper describes the *typical means used for transporting the meat,* with their relative capacities for the various cuts and their theoretical and real dimensions (by « real dimensions » is expressed the space which must be allowed per trolley in the chilling rooms):

A. — *Wheeled Trolley* (fig. 2) :

— length × breadth (150 × 100) cm = 1,5 m²
— real dimension = 3 m²
— Maximum load per trolley :

50 lombo Modena, 10 kg each = 500 kg
180 Coppe (trapezoid muscle), 2,7 kg each = 500 kg
50 Pancetta, 5 kg each = 250 kg
60 Lombo Bologna, 5 kg each = 300 kg
90 Boned shoulder, 5 kg each = 450 kg
Lardello (with skin) = 360 kg
Throat = 360 kg

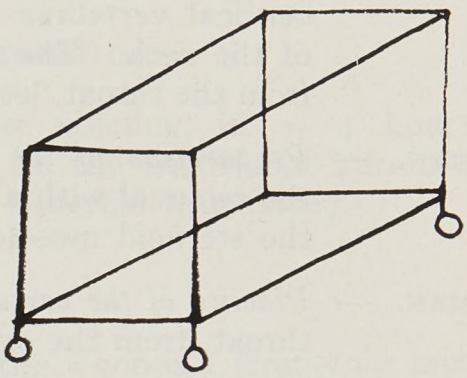

FIG. 2.

B. — *Stacking Tray* (fig. 3):

— the smallest lean parts are placed in layers not more than 8/10 cm thick;
— length × breadth (60 × 120) cm = 0,72 m²
— real dimension = 2,8 m²
— They can easily be stacked to a height of 10.
— Possible loads:

10 « trito » (small pieces lean meat) (25 kg each) = 250 kg
10 « carnetta » (small pieces of containing more fat) (25 kg each) = 250 kg
10 fat (40 kg each) = 400 kg
10 skin (40 kg each) = 400 kg
10 other parts (40 kg each) = 400 kg

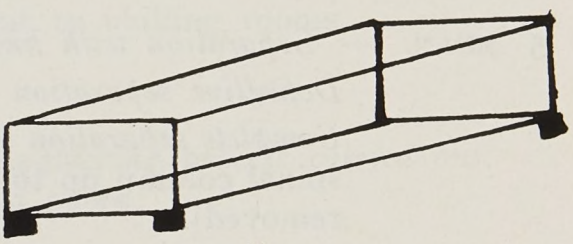

FIG. 3.

C. — *Car for overhead conveyor for hams* (fig. 4):

— each car can take 15 pieces;
— assuming that 2 hams stick out by 34 cm, We have:
car length × breadth (40 × 75) and thus = (40 × 34) × 75 cm = 0.55 m²;
— real dimension = 1.06 m²;
— each car occupies 1.6 m of the conveyor.

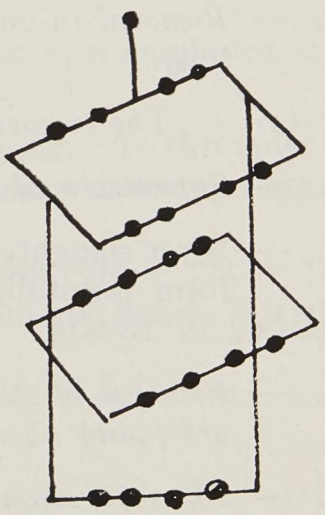

FIG. 4.

Description of cutting procedures

Manning levels

15-18 men are employed in the slaughtering procedures, depending on the operations being undertaken.

2 MEN. — *Removal of the head.* Carried out with a knife separating the first 2 cervical vertebrae (« atlante/occipitale »), then following the line of the neck. The head, hanging from a trolley, is then separated from the throat, leaving the cheek muscles on the bones of the head.

4 MEN. — *Preparation of the throat.* Skinning with an automatic machine, the removal with a knife of the lean parts of the rest (separation of the sternoid muscle from the parotid glands and their fat).

1 MAN. — *Placing of the carcase.* Removal of the carcase without head and throat, from the overhead conveyor, and placing on cutting bench.

1 MAN. — *Cutting of the ham.*

Cutting of the « coppa » with bone.

Using a circular saw the ham is cut following the line of the pelvis and the coppa with bone between the 4th and 5th dorsal vertebrae.

1 MAN. — *Removal of the « sugna » (perirenal fat).*

5 MEAN. — *Separation with knife of the ham.*

Definitive separation of the « coppa ».

Complete separation of the « lombo Modena » (comprises the ribs and spinal column up to the cut of the ileum where the ham has been removed).

Separation of the shoulders.

Apart from the separation of the shoulders the lean meat is cut from the « pancetta » (the oblique external muscle of the abdomen).

1 MAN. — *Preparation for skinning of the shoulders.*

2 MEN. — *Removal in one piece of the skin of the shoulders* (the phalanxes are left).

4 MEN (2). — *The removal of the soft fat from the shoulders.*

2 MEN. — *Separation of the « pancetta »* (side-as for bacon).

15 MEN. — After slaughtering and cutting, bring the « pancetta » to the correct form for italian processing.

(= 95 HOURS IN TOTAL).

2 MEN. — *Removal of the soft fat from the small lean parts. (The small parts are found above the adipose tissue of the spine and « pancetta ».*

3 MEN. — *The separation into component parts of the spine* (separation into skin, soft fat, lean parts and hard fat).

The operations described up to this point are carried out on the main bench. The following operations take place on the 3 lateral benches.

4 MEN. — *First lateral bench.*

Separation of the foot from the ham at the level of the metatarsal.

Separation of the lean which covers the side of the pelvis. Freeing of the upper end of the femur from the bone which covers it.

Separation following the vein of the fat of the ischio-pubis symphysis.

Hooking of the hams to the overhead cars.

19 MEN. — The day after slaughtering, after chicling, in ~ 4 hours for 800 hams, the hams are brought to the traditional Parma form. (Separation of adipose tissue and external lean parts).

(= 76 HOURS IN TOTAL).

8 MEN. — *Second lateral bench.*

Boning and cutting to shape of the « coppa » (trapezoid muscle).

The separation of the muscle from the ribs of the sternum.

9 MEN. — *Third lateral bench.*

Boning of the shoulders. Removal of bones (radius, ulna, humerus, scapula).

6 MEN. — 2 foremen, 4 men to carry meat to chilling rooms.

Considerations on productivity in the slaughtering process carried out on the lines outlined above

I shall consider one killing of 400 beasts carried out with a production line moving at 120 beasts per hour; at the same time however I shall consider the workforce employed for 4 hours in order to have real, not theoretical data.

As we see above, we have: 1) a maximum af 18 men employed in the killing.

2) a maximum of 59 men employed in the cutting.

Thus 77 men for 4 hours bring us to 308 hours of work. To this must be added the hours necessary later for the preparation of the hams and « pancetta » a further 76 + 95 hours = 171 hours.

For the complete preparation of 400 beasts we need 308 + 171 = 479 hours.

As we can see in the table of yields (table 1), from 400 beasts of 136 kg dead weight we obtain 55 400 kg of raw material:

Productivity is:

— Minutes per beast : 72.

— Kg /hour : 116.

Considering only productivity from the cutting of the carcase onwards, without considering the 18 men needed for the slaughtering, we have:

— Minutes per beast: 61.
— Kg /hour : 138.

I shall now present same data about the standard slaughtering and cutting process.

I shall take one killing of 400 beasts of 169 kg life weight (dead weight : 136 kg).

TABLE I

Anatomical cuts	Number of pieces per beast or carcase	Dead average weight kg	Type of trolley used N*	Real dimensions /Chilling room (m^2)	Total weight of cuts in kg
Lombo modena . . .	800	10.20	16 A	48	8 160
Ham	800	12.29	53 C	56	9 833
Coppe	800	2.72	5 A	15	2 177
Boned shoulder . . .	800	5.38	9 A	27	4 310
Small pieces of lean meat	400	4.23	7 B	6	1 673
Small pieces containing more fat	400	4.18	7 B		1 673
Pancetta (belly Bacon)	191	5.48	14 A	42	1 047
Pancetta (belly bacon)	609	4.32			2 626
Throat	400	4.57	5 A	15	1 828
Throat	400	1.30	2 A	6	520
Lardello (bacon fat)	400	17.66	20 A	58	7 063
Fat	400	19.33	20 B	60	7 733
Skin	400	2.24	3 A	9	897
Skin of shoulder . . .	800	0.475	1 B	3	380
Bone	400	7.39			2 956
Head	400	6.23			2 493
				345	55 400

N* the first figure is the number of transport units required.

Now let us examine the chilling conditions and bacterial levels of plate count for the principal cuts:

Ham (table 2)

Economically the most important item for Italian slaughterers.

The weight loss for hams of 14 kg to be cut to the definitive Parma shape is 1,5 per cent in 24 hours in the chilling room.

Taking the actual killing as a point of reference we have the following times:

Weighing of the carcase after 30 mn.
Separation of the ham after 35 mn.
Arrival in chilling room after 40/45 mn from the killing.

TABLE 2

Time in chilling room	Temperature of ham at centre (°C)	Temperature of chilling room (°C)	Relative humidity of chilling room
Empty chilling room . . .		— 5	79 /80
Entry of ham	40 /50	— 5	
After 2 hours.	29	+ 4	94 /94
After 3 hours.	24	+ 6	95
After 5 hours.	17	+ 3	95
After 6 hours.	14	+ 3	95
After 7 hours.	13	+ 2	95
After 8 hours.	12	+ 2	93
After 16 hours	5	0	78
After 24 hours	+ 1	0	75

Coppa (trapezoïd muscle) (table 3)

A characteristic local product for maturation.

Weight loss after 24 hours 2,27 per cent, taking the average weight of a coppa to be 2,7 kg.

Referring to the moment of killing, we have the following times for this cut, already boned on the production line:

Weighing as part of the carcase after 30 minutes.

Separation and boning after 38 minutes.

Arrival in chilling room after 128 /40 minutes from time of killing.

TABLE 3

Time in chilling room	Temperature °C of « coppa » at centre (°C)	Temperature °C of chilling room (°C)	Relative humidity of chilling room (%)
Empty chilling room . . .			
Entry of « coppa »	39 /50	— 5	79 /80
After 2 hours.	28	+ 9	94 /95
After 3 hours.	21	+ 8	92 /93
After 5 hours.	15	+ 5	94
After 6 hours.	13	+ 5	94
After 7 hours.	9	+ 6	95
After 8 hours.	9	+ 6	94
After 16 hours	1	0	84 /82
After 24 hours	— 1	— 2	80

Lombo modena (Table 4)

This is an anatomical cut destined for consumption as fresh meat. Average weight 10 kg.

Weighing of the carcase after 30 minutes.
Separation of the « lombo » after 35 minutes.
Arrival in chilling room after 60/40 minutes after killing.

TABLE 4

Time in chilling room	Temperature of « lombo modena » at centre (°C)	Temperature of chilling room (°C)	Relative humidity of chilling room (%)
Empty chilling room . . .		— 5	
Entry of « lombo Modena »	50/36	— 5	75/80
After 2 hours.	27	+ 9	94/95
After 3 hours.	20	12	94/05
After 5 hours.	15	5	94
After 6 hours.	13	5	94
After 7 hours.	12	5	94
After 8 hours.	10	6	94
After 16 hours	3	6	84
After 24 hours	0,5	— 2	80

TABLE 5

Time in chilling room	Temperature of boned shoulder at centre (°C)	Temperature of chilling room (°C)	Relative humidity of chilling room (%)
Empty chilling room . . .		— 5	75/80
Entry of boned shoulder	50/28	— 5	94/95
After 2 hours.	+ 11	+ 1	94/95
After 3 hours.	5	2	94/95
After 5 hours.	3	1	93
After 6 hours.	0	0	
After 7 hours.	0	— 1	
After 8 hours.	0	— 1	
After 16 hours	— 1	— 1	85
After 24 hours	— 1	— 1	75

Boned shoulder (Table 5) :

This is used for salami or mortadella.

Weighing of the carcase after 30 minutes.
Separation of the shoulder from the carcase after 39 minutes.
Arrival in chilling room after 90/45 minutes, after killing.

Top quality lean « Trito » (Table 6) :

This is used for raw salami.

Small pieces of lean meat obtained from various parts of the beast and various preparation processes = 4 p. 100 of the dead weight of the beast.

TABLE 6

Time in chilling room	Temperature of « trito » small pieces of lean meat at centre (°C)	Temperature of chilling room (°C)	Relative humidity of chilling room (%)
Empty chilling room . . .		— 5	75/80
Entry of « Trito » small pieces of lean meat. . .	36	— 5	94/95
After 2 hours.	11	+ 1	94/95
After 3 hours.	4	+ 2	94/95
After 5 hours.	1	+ 2	94/95
After 6 hours.	0	0	
After 7 hours.	— 0,5	— 1	
After 16 hours	— 1	— 1	85
After 24 hours	— 1	— 1	75

N.B. — The results relative to the first 4 hours are obtained during the filling of the chilling rooms. We should thus bear in mind what this implies.

Now let us examine the bacteriological condition of the cuts described above, using data from samples taken 24 hours after their arrival in the refrigeration room.

The figures given in the table 7 are the highest found in a year of monthly testing.

I also feel it is important to give the bacterial levels of typical mixtures for the making of Italian raw salami. The figures have been obtained from the analysis of mixtures of meat taken from cuts from the same abattoir and thus from meat of which, the bacteriological levels after 24 hours are those seen in table 7. The mixtures are usually prepared 3 or 4 days after the slaughtering of the meat, which is kept in the chilling rooms at 0/+ 3 °C.

TABLE 7

Anatomic cuts	Total plate count	Enterobacteriaceae	Coliforms	Clostridia	Micrococcus
Lombo modena . . .	9.2×10^3(*)	4×10^2	3×10^2	0	3×10^2
	5.5×10^4	6×10^2	4×10^2	0	3×10^2
Ham	8.5×10^3	8×10	5×10	10	2×10^4
	5×10^4	3×10^2	5×10	0	4×10^3
Boned shoulder . . .	9.5×10^3	10^3	5×10^2	0	7.5×10^2
	2.5×10^4	6×10^2	5×10	0	2×10^3
Coppa	6.5×10^3	2×10^2	10×10	10	8.5×10^2
	10^4	5×10^3	6×10^2	0	6×10^3
« Trito » small pieces of lean meat	8.5×10^4	2×10^3	10^2	0	6×10^3
	10^5	10^4	6×10^3	10	9×10^2

(*) N /gram.

TABLE 8

	Total plate count	Entero-bacteriaceae	Micrococcus	Lactobacillus
Salame « Milano » . .	1.5×10^6	5.1×10	1.5×10^6	5.6×10^2
Salame « Milano » . .	3×10^6	4.2×10	1.2×10^6	3×10^3
Salame « Felino » . .	6×10^6	1.5×10^2	2.1×10^4	2.4×10^2
Salame « Felino » . .	3.2×10^5	2.2×10^2	6×10^4	1.3×10^5

***

The boning while warm traditionally carried out in Italy continues to be normal practice for a number of reasons: in fact a technical link has established itself between slaughtering and cutting techniques and the characteristics of the traditional products.

To the economic motives for savings on the workforce, to which is connected the greater ease of preparing material while less hard, can be added numerous reasons of technical and commercial organisation linked to the features of the principal products.

The production of Italian pigs is orientated to the preparation of meat for products involving a maturation process and in particular to raw ham.

In the preparation of products which undergo maturation there is a tendancy to facilitate drying, i.e. the reduction of A_w. The principal factor in conservation and quality in the fermentation process is thus the neutralisation of the germs which could threaten the reduction of the pH.

In fact the presence of an acidic flavour or an excessive quantity of salt are both considered as negative factors.

The partial dehydration which occurs during the chilling of cuts which are still warm may imply on the one hand a reduced profit for the slaughtering organisation but provides on the other a better quality of meat for products requiring maturation. And operations are completed which would otherwise remain to be carried out during the later stages of processing of the meat.

The danger of the excessive proliferation of microbes represents in theory the greatest problem for the practice of boning while warm.

This necessitates a series of measures essentially linked to a production line moving relatively quickly in order to keep contamination levels within acceptable limits, even if it is not possible to have reliable data on the comparison between different techniques.

In fact we have no data on industrial processing carried out in the same way with meat obtained by the 2 different techniques. In any case the data on the contamination of meat boned while warm do not seem to reveal excessive dangers.

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Current status of electrical stimulation in New Zeland

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The use of electrical stimulation to accelerate the onset of *rigor mortis* and thereby reduce conditioning time is extremely important to the New Zealand meat industry. This is especially true for the export lamb trade dealing with large numbers of lambs which are frozen soon after slaughter. Until recently it was common for lamb carcasses to be moved into freezers within 2 hours of slaughter and be frozen to — 18 °C within another 10-14 hours. Muscles of these carcasses cold shorten during the early phases of freezing and are totally frozen before *rigor mortis* is complete. If cooked from the frozen state the muscles undergo thaw rigor and shorten further but if thawed slowly prior to cooking exhibit only cold induced shortening. Cold and thaw shortening, either separately or in combination cause meat to be tough (Marsh *et al.*, 1966). Electrical stimulation of lamb accelerates the onset of *rigor mortis* so that the period when the muscles are susceptible to cold shortening is greatly reduced. The faster rate of glycolysis also reduces the time for ATP reserves to be metabolised reducing the period which must elapse before freezing if thaw rigor is to be avoided.

The large numbers of lambs to be handled in a short time require that any stimulation process be automatic and simple. A simple rubbing contact was considered possible but presented two related problems for the stimulation of wooly lambs immediately after slaughter. Firstly, dry wool provides a high resistance path between a rubbing electrode and the carcass. However, the resistance may be modified by changes in length of wool and degree of wetness. Secondly, in order to ensure equal stimulation of all lambs passing across the electrode either, all had to have equal resistance, or a segmented electrode system with a power supply which could overcome the initial high resistance, and then, supply the required peak current was required. Although use of high pressure water jets significantly reduces contact resistance there is still considerable variability. Use of segmented electrodes each supplied with its own current controlled power supply ensured that every carcass passing accross the electrode received approximately the same degree of stimulation. High voltages were used but as current flowed the voltage collapsed to give a 2 amp peak current of 14.28 pulses per second, polarity reversing after every pulse. Early studies conducted with the carcasses hanging from one hind leg showed that the leg which was not in the direct current

path was not as tender after stimulation treatment as the directly stimulated leg (CHRYSTALL, 1976). A system in which both hind legs were used as electrical contacts was developed and this resulted in equal tenderness in both hind legs. The 60 s stimulation, gave maximal reduction in time to rigor onset but caused dressing difficulties in that the carcasses became too stiff early in the operation. However with a slight increase in time from stimulation to freezer entry, 45 seconds was found to be satisfactory and did not cause early stiffening.

Stimulation of carcasses after dressing was simpler in that all carcasses are suspended from a gambrel between the hocks of the hind legs and the naked carcasses proved a relatively uniform electrical contact so that a single electrode could be used to stimulate all the carcasses. The falling temperatures and neural

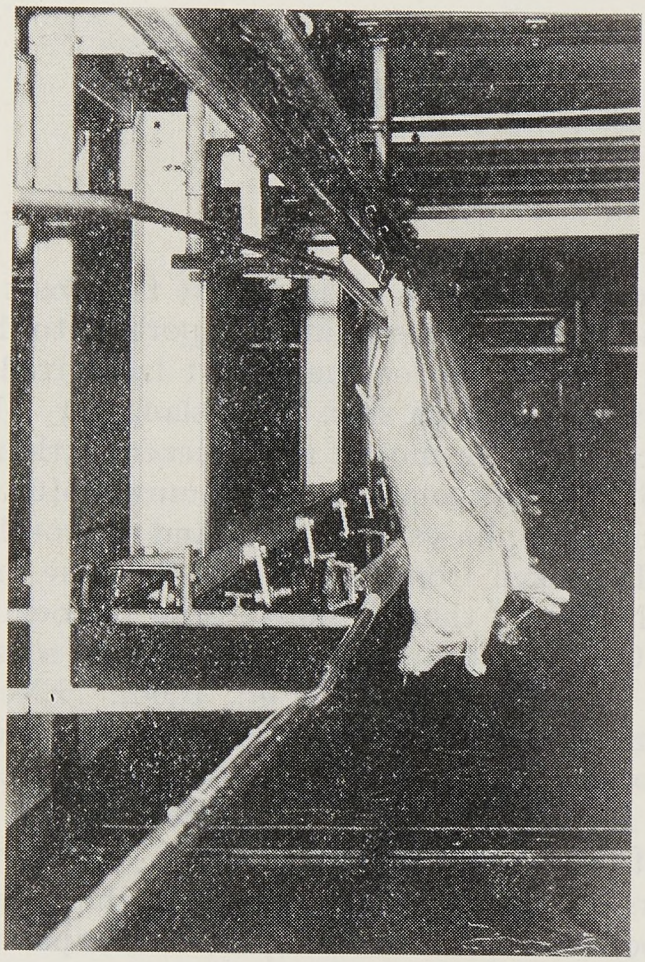

FIG. 1. — *Post dressing electrical stimulation facility for lambs handling 32 lambs per minute on one electrode*

death places a limit on the period when carcasses are responsive. Initial trials using a 14.28 pulses per second waveform derived from a 400 V (RMS) 50 Hz supply showed that a 90 sec stimulation period up to 30 minutes *post-mortem* gave satisfactory tenderness results for the loins but the legs were tougher than desired. Use of pulses derived from 800 V (RMS) 50 Hz supply slightly improved loin tenderness and greatly improved tenderness of the legs (HAGYARD *et al.*, 1980, CHRYSTALL 1978).

Stimulation, is however, just one step of an 'Accelerated Conditioning' process which ensures production of moderately tender lamb regardless of whether it is cooked from the frozen or thawed state. Stimulated lamb carcasses are required to dwell at not less than 6 °C for at least 90 minutes after stimulation and

then subjected to a controlled freezing rate. An aging period is added if a greater degree of tenderness is desired for certain markets. Deviation from the specified processing conditions results in a change in tenderness of the product. An important aspect of ,, Accelerated Conditioning '' is that it was developed to ensure a product at least as tender as lamb held for 24 hours at 10 °C before freezing. It is not merely to get any tenderness improvement.

A Quality Assurance program has been developed to ensure that product, meets the tenderness requirements set down for Accelerated Conditioned or Acce lerated Conditioned and Aged product. For use in the quality assurance program Meat Industry Research Institute of New Zealand (MIRINZ), developed

FIG. 2. — *The original predressing electrical stimulation tunnel with a segmented electrode system*

a simple pneumatically operated tenderometer which gives tenderness results which are directly comparable to those on the Mirinz tenderometer (MACFARLANE and MARER, 1966).

At the present time, i.e. commencement of the 1980-81 killing season stimulation equipment has been installed in over 80 p. 100 of all lamb export plants so that almost 90 p. 100 of all lamb carcasses is being stimulated. In other words 22 million lambs are now being stimulated annually. The majority of companies have elected to use post dressing stimulation systems (fig. 1), with one tunnel, enclosing one or two electrodes. Each electrode being designed to stimulate 24 to 32 lamb carcasses per minute although one installation has been designed to stimulate 48 lamb carcasses per minute on a single electrode. Some companies have installed the predressing system with one tunnel for each dressing line (fig. 2).

Since in both predressing and postdressing electrical stimulation systems we have elected to use high voltages in order to get maximum degree and uniformity of effect, safety is paramount. In conjunction with electrical supply authorities, MIRINZ drew up recommended safety guidelines (MIRINZ, 1978). These lay down the minimum safety requirements for electrical stimulation systems in New Zealand. To ensure that entry into an operating stimulation tunnel is virtually impossible except for carcasses on the conveyor rail, pressure foot plates at entry and exit ports are wired with double switches to shut off power if entry is attempted. Light beams are used as a second line of safety protection. Some tunnels are not straight so it is impossible to ensure they are clear without physically checking the entire length. Key switches, strategically placed throughout the tunnel and at entry and exit points, which must be activated in a set sequence ensures the tunnel is cleared during initial set up. The safety circuits must be tested in open and closed positions before the high voltage supply can be energized.

If the safety circuits are tripped the whole start up process must be repeated. Although likely to err on the side of caution the guidelines have been accepted within the New Zealand context so that even with the high voltages, stimulation is safe.

Commercialization of beef stimulation has been slower although the first commercially operating beef stimulation unit was in New Zealand. Much of New Zealand beef is exported as boneless manufacturing beef for which electrical stimulation is no advantage. However a number of companies exporting prime beef cuts are currently negotiating with equipment suppliers for stimulation systems. The main advantages they see are the possibility of avoiding cold shortening (DAVEY *et al.*, 1976), induced toughness regardless of chilling rate, and the reduction of aging time that is possible with earlier onset of *rigor mortis*. MIRINZ has issued specifications to the New Zealand industry, covering stimulation of beef as hide on or hide off whole bodies, eviscerated carcasses or as sides. Electrical stimulation has been successfully used as an adjunct to hot boning of bobby calves and mature beef.

Electrical stimulation systems for lamb and beef are supplied by:

— McKenzie and Holland Limited
P.O. Box 35-097, Naenae (New Zealand)
— Thornton Engineering Limited
P.O. Box 22223, Otahuhu (New Zealand)

Both companies supply complete systems including power supply, safety circuitry and electrodes. Although circuitry is different, both power supplies deliver 14.28 pulses per second with alternating polarity. For systems designed for the post dressing stimulation of lamb or for the stimulation of beef, 800 V (RMS) transformers are used with power rating depending on the likely maximum load. The higher voltage system used for predressing stimulation of lamb is supplied by McKenzie and Holland Limited.

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Co-operation between industry and research in developing a method for production of hot boned, primal cuts of beef

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Summary

For more than two years Celebrity Food Factories have had a full scale production of hot boned, vacuum packed Primal cuts of beef. The Danish Meat Research Institute participated in investigating and developing the production techniques.

The factory developed all cutting techniques, packaging methods etc., while the Institute assisted by investigating hygienic aspects and by optimizing the chilling programmes.

The outlines of results obtained during the initial investigations as well as results and experiences gained, after the production has been established, are described.

The conclusion of the work is that a successful production of hot boned beef is possible when the slaughter— and cutting hygiene is kept at a high standard, and when the chilling conditions are rigidly controlled.

Introduction

Earlier in this seminar a description has been given of the actual production of hot boned, vacuum packed primal cuts of beef. During the development of the methods for this production on the Celebrity Food Factories, as well as during the early stages of the production itself there has been a close co-operation between the Celebrity Food Factories and the Danish Meat Research Institute. It is the intention of this paper to describe the background and results of this collaboration and thereby illustrate some of the areas, which in our opinion, are crucial to a successful production of hot boned meat.

The start

In the spring of 1976 Celebrity asked the Institute to assist in undertaking investigations on hot boning of beef. The factory had been inspired by a description in the Institute's newsletter « Orientering » on modern trends in the New

Zealand meat industry written by guest researcher S. L. Oldfield, Massey University. Based on this description Celebrity had undertaken some preliminary investigations and found them to be so promising, that they wanted to continue.

The Institute was well equipped for this task, because it had a solid knowledge of the influence of different chilling rates on the tenderness in beef (BUCHTER, 1972). The idea of hot boning was well known, partly from the original paper on hot boning of beef by SCHMIDT and GILBERT (1970) and partly from communications with M. J. FOLLET (FOLLETT *et al.* 1974), who developed the method commercially for J. SAINSBURY Ltd., and who on a personal basis had consulted the Institute concerning the influence of chilling rates on tenderness in beef. Finally, the technology involved in ageing and storage of beef in vacuum bags was well established commercially.

The financial background for the co-operation between Celebrity and the Institute was as follows:

The Danish Meat Research Institute is owned and financed by the industry. Research projects which are of common interest, and from which the results will be available for all members of the industry, can be financed by general funds collected from a slaughter-levy. Projects which are of specific interest to a single factory are, however, to be paid directly.

The Danish Livestock and Meat Board found the aspects of hot boning to be of general interest to the whole industry and they decided that the research should be financed by them.

The work was divided between Celebrity and the Institute. Celebrity was to develop all practical aspects such as cutting techniques, packaging method, etc., while the Institute was to investigate the hygienic aspects and optimize the chilling methods.

Investigations

The first investigations included four series of 6 commercial cows and dealt only with the cuts in the hindquarter (coupe « pistolet »). From the first 18 cows one side was traditionally chilled and the other was hot boned. For the last 6 cows both sides were hot boned.

The experiment was carried out on a Celebrity beef plant and the boning was performed by ordinary workers. The hot boning took place within 1½ hrs. *post mortem*. All cuts were immediately vacuum packed, placed in single layers on shelves and exposed to different chilling programmes.

The traditionnally chilled sides were cut 3 days after slaughter and the cuts were vacuum packed and stored together with the hot boned cuts at 2 °C until approx. two weeks after slaughter, when the majority of evaluations took place. Further details of the experimental design is given by BUCHTER (1977).

Results of yields and of pct. drip loss are shown in Table 1. It can be seen that hot boning gave higher yields of whole cuts and lower drip losses in the vacuum bags.

The results of taste panel evaluations and consistency measurements which are also shown in detail by BUCHTER (1977) indicated clearly, that especially the small cuts could become very tough when they were chilled too fast. If the cuts, however, were chilled more slowly, the average tenderness in the hot boned meat would be very close to the tenderness found in the traditionally chilled sides.

Under the conditions used in the investigations optimal tenderness was found

TABLE I

Comparison of yields and pct. driploss from traditionally chilled and hot boned beef (18 cows)

Cut (name of muscle)	Yield in kg		Drip loss in pct.	
	Traditionally chilled	Hot boned	Traditionally chilled	Hot boned
Fillet (psoas major) . . .	2.0	2.1	1.1	1.1
Striploin (long. dorsi) . .	4.6	4.8	1.0	0.8
Entrecote (long. dorsi) . .	2.4	2.7	—	—
Rump fillet (gluteus medius)	3.1	3.5	2.3	1.3
Culotte (tip of biceps fem.)	1.5	1.4	1.1	0.8
Silverside (semitendinosus)	2.3	2.4	1.0	0.2
Topside (semimembranosus)	8.6	8.8	0.7	0.5
Toprump (biceps femoris)	5.0	5.3	—	—
Thick flank (quadriceps)	4.9	5.2	—	—
Total cuts	34.5	36.3		
Trimming meat	14.1	13.0		
Tallow	5.1	4.5		
Bone	13.7	14.9		
Cutting loss	0.1	0.1		
Weight of hindquarter	67.4	68.8		
Chilling loss	1.4	0		

to occur when the cuts were conditioned approx. 7 hrs. at 15 °C before they were further chilled at 7 °C. 24 hrs after slaughter all cuts were transferred to 2 °C.

The experiments also showed that the keepability of the hot boned cuts was satisfactory. Normally vacuum packed cuts from the traditionally chilled sides would smell slightly fresher. However, when the initial bacterial counts were low i.e. when the slaughter— and cutting hygiene was good, all hot boned cuts would still be fresh after 6 weeks of storage at 2 °C. For retail packed beef steaks it was found that the hot boned meat had better colour stability.

A second series dealing with hot boning of forequarters gave similar results. The types of cuts which could be obtained by hot boning of forequarters were, however, very different from the cuts usually obtained from forequarters.

All in all, the investigations convinced Celebrity, that the idea of hot boning beef was worth while pursuing.

Preparing for production

Right from the start of investigations, the Institute had pointed to the following three basic rules which had to be followed if hot boning was to succeed as a production method:

1) Only animals which would not develop DFD could be hot boned.

2) The slaughter and cutting hygiene during production of hot boned meat should be of a high standard, and it should be controlled by frequent bacteriological analyses.

3) The temperature during chilling and storage of the primal cuts should be rigidly controlled.

All three measures where to ensure a production of a high and uniform quality both regarding bacteriological and meat quality aspects:

1) This would give no difficulties because Celebrity only wanted to hot bone cows, and according to the Institute's experience DFD-problems are seldom met in cows.

2) It is evident that hot boned meat is more succeptible to contamination than traditionally chilled meat, if care is not taken. Throughout the investigations as well as during the actual production, Celebrity has been able to keep a good hygienic standard, which is controlled through daily analyses of the total viable counts on carcasses and cutting tables.

3) The chilling programmes which can be used for hot boned, vacuum packed primal cuts will represent a narrow balance between considerations of hygiene and of meat tenderness. As mentioned earlier the investigations showed that great care had to be taken if cold shortening, especially in the small cuts, was to be avoided. Too slow chilling will, however, provide possibilities for unwanted bacteriological growth. The philosophy behind choosing to chill the vacuum packed cuts in thin layers on shelves has been to provide as uniform a chilling of all surfaces as possible.

During 1977 Celebrity prepared to start production. The Institute helped in the negotiations with the Ministry of Agriculture and the Veterinary Authorities about obtaining permission for a production of hot boned beef. The outcome was that Celebrity obtained a provisional permission, and at the same time it was agreed that the Institute in co-operation with the Veterinary Authorities should follow the hygienic standard and chilling conditions.

In the Autumn of 1977 a team of unskilled workers, who had never cut meat before, was trained for hot boning at Celebrity by the Danish Meat Trade School. Again the Institute followed the hygiene and chilling. At this time the modernization of the slaughter line and the cutting room at Celebrity's Rødding plant was well underway and by April 1st, 1978 the production started.

Assistance during production

The programmes for routine control of slaughter— and cutting hygiene were set up by the Institute. The method used was the « Swab /Agar slope method » which was developed and described by ØLGAARD (1977). This method is a simple rapid method which has proved itself to be suitable for this type of investigation. The tests are performed by Celebrity's own laboratories and the results are reported to the Veterinary Authorities and to the Institute.

Right from the start, the factory has understood the importance of good hygiene. Thus, when the new slaughter line was first manned the number of slaughter animals was kept very low so that each work position could be carefully

adjusted to allow the best possible working conditions and hygiene. Even after full production was obtained the work speed and the incentive payment system have been regulated to allow each worker ample time to follow his work instructions in detail. This has proved to be effective. The total bacteriological counts on the carcasses, which were extremely low during the first three months of production increased slightly and then stabilized. On and off the hygiene has had to be tightened. But this has so far automatically been taken care of by Celebrity itself.

The workers are well aware of the importance of hygiene and they take pride in the production. The workers also know that the plant can be closed if the Veterinary Authorities find the conditions not to be satisfactory. The work of improving slaughter hygiene is a never-ending one. Only recently a small alteration in the method of dehiding has resulted in lower bacterial counts on the top rounds.

The chilling conditions have been monitored and followed constantly since the start of the production. The Institute has helped in this task and has also adjusted and optimized the chilling programmes to follow alterations in production conditions for the hot boned primal cuts. Thus, under practical conditions it has proved to be possible to reduce the temperature and the time of the conditioning and still chill the vacuum packed cuts without risks of cold shortening. At the start the cuts were chilled in existing chillers which were equipped with the necessary thermostats and automatic time controls. Lately new, completely automated chilling rooms have been built which are fitted to handle larger quantities and at the same time allow a better flow of the production.

Investigations after the start of production

Hot boned meat is a new commodity and its performance under different conditions is closely followed by the Danish Veterinary Authorities.

In theory vacuum packed cuts of hot boned meat can develop a bacterial flora of a different composition than the one normally found on vacuum packed cuts from traditionally chilled sides.

The Veterinary Authorities have themselves undertaken some preliminary experiments to see whether pathogenic bacteria could be a risk under the production conditions used at Celebrity. This was found not to be the case.

The Institute has undertaken investigations using the hot boned meat for different types of retail packaging. One of the latest in which the hot boned primal cuts were used for producing controlled atmosphere (80 p. 100 oxygen and 20 p. 100 carbon dioxide) retail packs have been reported by BUCHTER (1980). None of these investigations have indicated that the hot boned meat gives any bacteriological problems.

Recently an investigation over the use of el-stimulation has been carried out at the Rødding plant. The experiments which have been undertaken in co-operation with the Swedish Meat Research Institute are still not finished, but the results obtained so far do not indicate that electrical stimulation gives better meat quality than the one obtained in Rødding by the present system.

Closing

As a research worker it has been extremely inspiring to work directly in co-operation with an industrial plant, and to see the work lead to a major development in the pattern of the cutting and distribution of beef. In retrospect the success of this new production has been based on three factors:

1) A management who had the vision of innovative production forms as well as the ability to analyse and judge when and where such productions could succeed.
2) A production leader who put his « soul » in developing the new techniques and in making these techniques function in the production situation.
3) Support from a research institute to optimize the production conditions and thereby ensure the quality of the product.

Of these three the last might have been the least necessary. I do, however, feel that research has also played an important part, because it has provided the proof of the quality of the products and thus made it easier for the factory rightfully to negotiate with the financing boards, the Veterinary Authorities, the workers and the customers.

Celebrity Food Factories took the initiative and had the possibilities to realize the production of hot boned beef first. As a closing remark it should, however, be remembered that the research was generously paid for by the whole Danish Beef Industry.

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Apparatus for electric stimulation of hot boned meat cuts

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Summary

An apparatus for electric stimulation of hot boned meat cuts in order to obtain accelerated conditioning and thereby prevent cold shortening is described.

The main idea is to use the apparatus for stimulating only the hot boned cuts which are going to be eaten as whole meat pieces and not the trimmings which are going to be used as raw materials for sausages, ground beef and other products where a high water binding capacity is desired (Beehive extruder and Urschel machines).

By stimulating only the hot boned cuts and not the whole carcasses, it is possible to use a *very* low voltage which makes the equipment safer for the operators than equipment designed for accelerated conditioning of whole carcasses.

The stimulation is carried out on the meat after the point in time when electric stimulation is effective through the nervous system. The electric stimulation works on the muscles directly and not through the nerve ends which may be already blocked 30 minutes *post mortem*.

Too high voltage may give an dielectric heating effect on the meat. This may easily be avoided by operating at low voltages which at the same time causes nohazar ds to the operators.

The hot boned trimmings may be used directly for chopping or they may be frozen rapidly and stored below — 20 °C for use later as raw material for sausages. They must be chopped in a frozen state in order to keep their excellent water holding capacity, it not satted (2 %).

The hot boned accelerated conditioned cuts may be packed and chilled normally and then brought directly into distribution.

Introduction

It has been known for many years that electricity may be used for accelerated conditioning of carcasses (fig. 1) to prevent cold shortening during chilling. Cold shortening gives what might be called « cold toughening ».

It has also been known for many years that prerigor meat has a very high water holding capacity compared to postrigor meat (40 %).

The water binding capacity of meat from electrically stimulated carcasses is estimated to be approximately the same as the water binding capacity of normally

chilled postrigor meat. More research may perhaps here show results in favour of normally chilled non-stimulated meat.

The development of the Norwegian equipment for stimulation of hot boned meat cuts is based upon the above mentioned facts. The main idea is to stimulate only the cuts and not the trimmings and thereby let the latter keep their « prerigor » advantages.

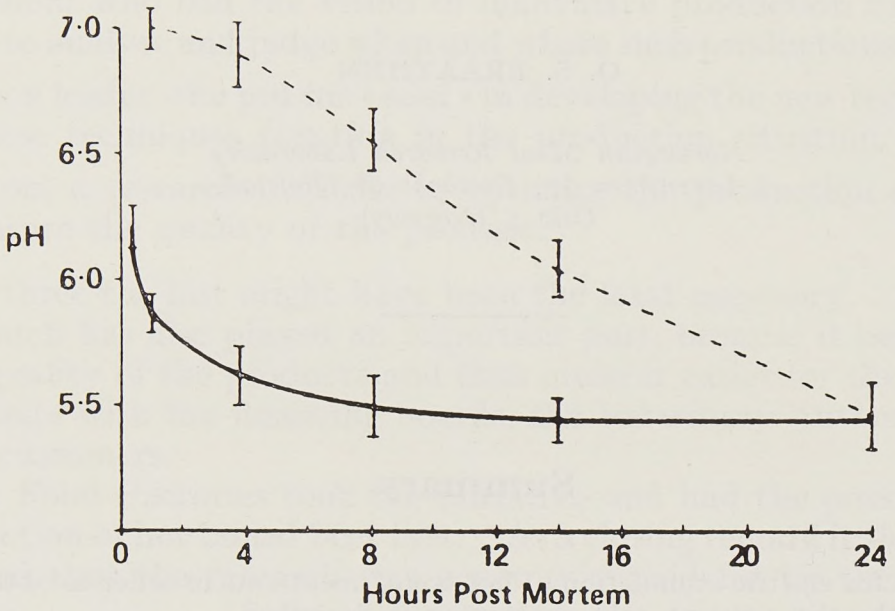

FIG. 1. — *Time course of pH fall in longissimus dorsi muscles of stimulated* (——) *and unstimulated* (- - -) *animals. Standard deviation shown by vertical bars. (Adapted from Chrystall and Hagyard, 1976).*

Laboratory work

During the start of the research on development of the apparatus, laboratory tests were carried out using an electric prod which is normally used for making animals move forward to the killing floor. It was shown that the electric prod made cuts of meat contract even after a relatively long time from slaughter: two hours.

It was then decided to make an apparatus for pilot plant tests.

Pilot plant apparatus

During the initial development period it was estimated that quite high voltage should be used to give contraction in the meat. Very high voltage was seen in practical operation by the author in slaughterhouses in New Zealand during a visit in 1976. In those installations approximately 3 000 volt were used for stimulating lambs immediately after exsanguination. The effect on the lamb carcasses was excellent and buying companies asking for New Zealand lambs with « conditioned quality », got what they expected.

Strict safety precautions were taken into consideration during the design of

the apparatus and were based on the following: Any voltage that gives effective stimulation of a carcass to prevent cold shortening might also be « effective » on an operator. The main reason was supposed to be that a stimulating voltage might give an undesired stimulation of the breathing muscles for a longer period of time so that the operator may not get enough oxygen. It was soon found that the very sophisticately developed plastic protection hood was not necessary. This was because the voltages necessary for stimulating hot boned cuts were much lower than those necessary for stimulating a whole carcass at the same time after stunning. The construction of the pilot plant apparatus was to a certain extent unnecesary complicated because different high voltage safety units were built into it.

Safety micro-switches were built into the plastic hood or lid of the apparatus.

The whole conditioning process or electric stimulation took place inside a plastic hood measuring 50 × 50 × 50 cm.

A transparent plastic cover was used so that the cuts of meat might be seen while contracting during stimulation.

Square metal plate electrodes

During stimulation the piece of hot boned meat was first put on a square stainless steel buttom plate electrode.

A similar plate was used as an upper electrode. These electrodes measured 30 × 30 cm.

When the voltage from a special electric pulse generator was applied a rapid contraction of the cut of meat took place.

The stimulation generator

The « power supply » for the stimulating unit was developed by the company Scankontroll A /S, Bleikeråsen 217, 1370 Asker, Norway. It was easily regulated to give different voltages.

Different types of pulse forms as well as stimulating frequences could be generated and applied to the meat cuts.

Excessively high voltage may give dielectric heating

When excessively high voltages were applied to the cuts of meat, the meat contracted normally during a « normal » interval. But a very high voltage also produced heating of the meat, giving a grey discoloration.

The longer an excessively high voltage was applied, the warmer became the cut of meat. The grey discoloration was seen where the contact electrodes were placed. When experimenting with a smaller contact electrode a local grey discoloration and a « burned » flavour developed.

Continuous or revolving unit

Originally it was meant that a carucell based system might be used for the stimulation process (fig. 2). Later it was decided to build a unit which instead of turning around a vertical axle, turned around a horizontal axle (fig. 3).

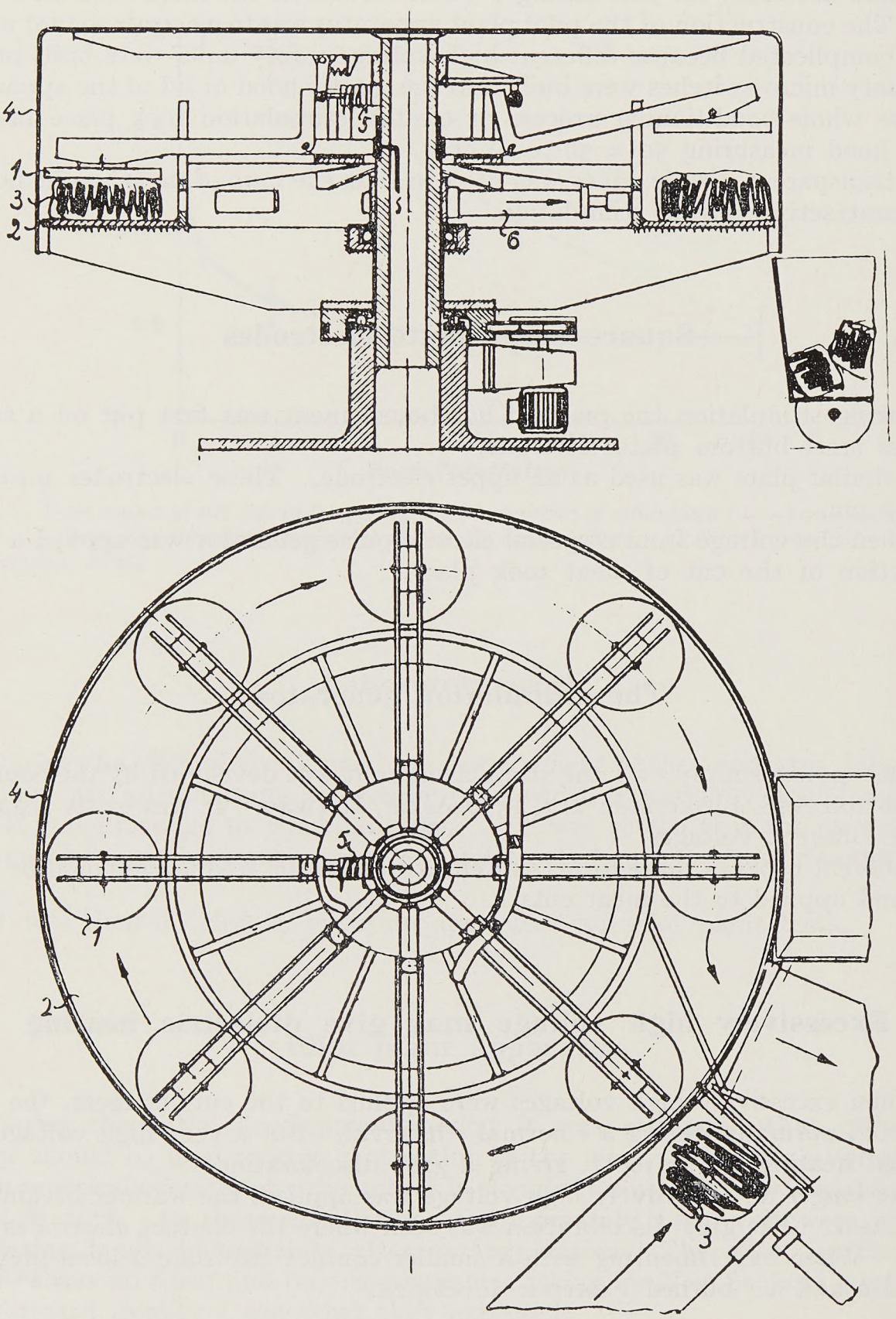

FIG. 2. — *Revolving unit for electrical stimulation.*

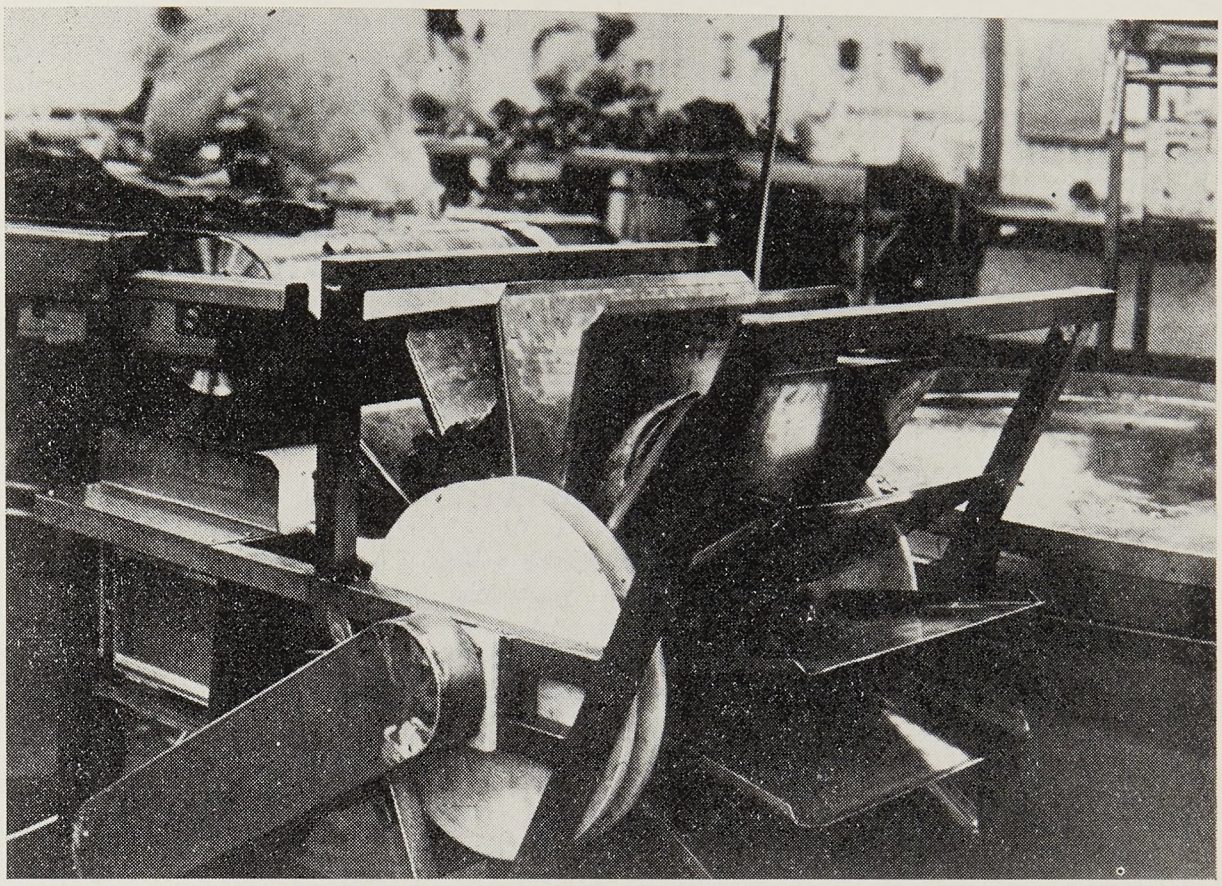

FIG. 3. — *Apparatus for stimulation*

The unit is meant to be put on the end of the meat cutting table and the hot boned pieces of meat to be eaten as such are run through the apparatus.

Taste panel tests showed that the tenderness after storage was better on the meat cuts having been stimulated. The pH-drop in the meat one hour after stimulation showed a difference of about 0,7 pH-units.

The apparatus is still under development by the company Landteknikk A /L, Persveien 28, økern, Oslo 5, and is shown on figure 3.

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Stimulation électrique des carcasses, désossage à chaud des viandes

Aspect réglementaire

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Actuellement, il n'existe pas de dispositions réglementaires spécifiques dans le domaine national, en ce qui concerne ces deux opérations.

Alors que le désossage des viandes, en raison des manipulations qu'il nécessite peut constituer, s'il n'est pas rigoureusement effectué, une source de contamination importante pour les produits obtenus, la stimulation électrique semble n'avoir aucune influence sur ce processus.

La stimulation électrique peut être employée seule, selon des conditions techniques parfois très différentes, pour obtenir par exemple une diminution du temps de maturation des carcasses; lorsqu'elle est associée au désossage à chaud des viandes, elle permet, tout en respectant les obligations communautaires du ressuage rapide des viandes, de garder aux pièces de viandes désossées toute leur qualité organoleptique, notamment d'éviter plus ou moins le phénomène du « cold shortening ».

1. — La stimulation électrique

Les réglementations communautaire ou nationale ne font pas état de cette pratique.

En l'état actuel de nos connaissances, il semble bien que rien ne s'oppose à son utilisation sur le plan de l'hygiène. Les études expérimentales actuellement menées par différents organismes permettent d'envisager et de fixer les limites dans lesquelles cette technique pourra intervenir :

— précocité de la stimulation;

— lieu de l'action électrique;

— possibilité ou non de stress et répercussion sur les milieux intérieurs et les muscles, etc...

2. — Le désossage à chaud des viandes

La Directive C.E.E. du Conseil du 26 juin 1964 modifiée, concernant les échanges de viandes fraîches de boucherie, a prévu ce mode de désossage (annexe I, chapitre VII — point 34).

Il est bien certain que dans la mesure où cette forme technologique nouvelle viendrait à se développer en France, l'élaboration d'un texte particulier, venant compléter l'Arrêté ministériel du 18 juillet 1977, deviendrait nécessaire.

En effet, les conditions techniques actuelles du désossage à chaud, afin de garder aux viandes leurs qualités organoleptiques optimales, se heurtent aux prescriptions de la Directive susvisée, et notamment, à l'obligation d'utiliser précocement le facteur limitant constitué par la réfrigération des viandes.

Cette Directive, dont le respect est inévitable si les viandes découpées veulent bénéficier de l'estampillage communautaire, impose :

— le traitement continu des carcasses depuis l'abattoir jusqu'au local de découpage sans solution de continuité;

— le travail des viandes à un pH compris entre 5,6 et 6,1;

— un traitement immédiat par le froid des pièces découpées.

Ces obligations supposent évidemment un aménagement particulier des locaux, qui doivent se trouver obligatoirement en annexe de l'abattoir, et afin d'éviter toute rupture de charge, disposer d'un réseau aérien conçu de telle sorte que les carcasses destinées à ce type d'opération passent directement, sans ressuage, du hall d'abattage à l'atelier de découpage.

Or, ces différentes mesures ne sont pas compatibles avec le désossage à chaud, tel qu'il est préconisé actuellement pour conserver aux pièces découpées leur tendreté; notamment par la mise en attente des carcasses sans réfrigération à des températures supérieures à + 7 °C. C'est pourquoi, la mise en application sur les carcasses à l'abattoir de la stimulation électrique, en permettant d'obtenir rapidement un abaissement de pH de la viande et ainsi accélérer le déclenchement précoce des phénomènes de maturation dans les muscles, apporte la solution aux problèmes soulevés. Il permet une réfrigération rapide des carcasses et des viandes, seule possibilité de supprimer ou de limiter considérablement les risques de contamination microbienne.

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La stimulation électrique et le désossage à chaud : Conséquence sur l'agencement de la filière viande

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Si j'ai bien cerné la pensée des organisateurs de ce colloque et compris le sens attribué à ce sujet, il fallait un intervenant « d'aval » des scientifiques, technologues et hygiénistes pour traiter des implications techniques ou technico-économiques de l'utilisation industrielle des deux nouveaux procédés en question et des aspects essentiellement ingéniérie de leur mise en œuvre dans des établissements d'abattage, de découpage et de conditionnement des viandes fraîches en l'état, donc en réalité dans la seule partie moyenne de la « filière ».

Le sort est tombé sur le C.T.G.R.E.F. Or, à ce jour, les Divisions « Viande » et « Froid » de ce Centre technique public, si elles ont été à l'écoute des informations et connaissances nombreuses, diverses, souvent approximatives, parfois peu concordantes, sur la question et si elles ont commencé à réfléchir sur les conséquences de l'application de l'électrostimulation (E.S.) et du désossage à chaud (D. C.) dans la conception et la réalisation des outils industriels futurs, elles n'ont pas encore véritablement engagé les études de leur compétence sur ce thème.

Vous comprendrez dans ces conditions que mon propos ne peut aujourd'hui être très sûr et qu'il peut apparaître sommaire ou schématique. Il a surtout pour ambition de servir de base à une réflexion, en posant les problèmes dans des structures d'entreprises et un contexte qui sont ceux de la France... et en espérant que tout n'a pas déjà été dit en cette fin de colloque.

Il n'y sera pas question du travail des porcs, qui ne sont pas justiciables manifestement de la stimulation électrique.

De plus, on peut s'interroger sur l'intérêt du développement généralisé de l'électrostimulation aux ovins dans la mesure où ils ne sont pas ou peu expédiés au loin, suivant une filière très intégrée de l'abattage à la distribution, notamment sous forme congelée comme en Nouvelle-Zélande, ni découpés en ateliers industriels, sauf dans quelques cas spéciaux, à notre connaissance, encore rares.

L'orientation moderne paraît bien être de concevoir dans ce pays et pour cette espèce des abattoirs équipés de lignes d'abattage et d'équipements frigorifiques particuliers permettant une cinétique du refroidissement des carcasses d'agneaux mieux adapté que ce qui existe actuellement dans des abattoirs plus ou moins « polyvalents ».

Seul le travail des gros bovins sera examiné en définitive.

1. — Le processus actuel

Les innovations technologiques en question doivent s'appliquer :

— l'une, la stimulation électrique, au stade de l'abattage, qui correspond à une « première transformation » de l'animal en viandes (carcasses et abats);

— l'autre, le désossage à chaud, au stade de la « deuxième transformation », qui consiste à découper les carcasses (entières, en demis ou quartiers) et conditionner les produits plus ou moins élaborés en résultant.

Progressivement, à chacun de ces stades a été utilisé le froid artificiel sous forme de réfrigération ou de congélation et cela a été la première révolution de la filière viande.

A l'origine et majoritairement encore, les manipulations et opérations relevant de la première transformation s'effectuaient à l'abattoir « sensu stricto » : abattoir public plus ou moins « polyvalent », abattoir de statut coopératif ou privé spécialisé dans le travail d'une ou deux catégories d'animaux, l'expédition des viandes à partir de ces établissements étant réalisée en carcasses le plus généralement réfrigérées.

Aujourd'hui, les files et chaînes modernes françaises ont une capacité d'abattage allant de 15 /20 bêtes /h jusqu'à 80 /90, ce qui correspond, dans le cas d'approvisionnement et d'exploitation rationnels, à des abattages annuels de 7 000 à 40 000 t de gros bovins.

Les opérations de découpe, avec ou sans désossage, et de conditionnement-emballage s'effectuaient dans des établissements industriels ou semi-artisanaux, généralement distincts, très souvent éloignés des premiers, les carcasses étant transportées jusqu'à ces ateliers sous température dirigée.

La recherche constante de meilleures conditions d'hygiène, de qualité et de productivité dans les abattoirs et les industries connexes, certains développements et améliorations techniques, la transformation accélérée au cours de la dernière décennie des circuits de distribution et commercialisation et des professions du secteur, ont amené à la situation actuelle en France : concentration des centres d'abattage, implantation sur les lieux de production et, de plus en plus, dans la dernière décennie, association sans solution de continuité, des ateliers de découpe-conditionnement puis, le plus généralement, transport toujours sous régime de froid des produits de découpage tels que pièces parées prêtes à trancher (P.A.T.) et conditionnées sous vide, vers les lieux de consommation où elles sont portionnées et reconditionnées en unités consommateurs, notamment dans les centrales ou grandes surfaces de la distribution moderne, avant leur commercialisation au détail et à court terme.

C'est cette organisation moderne de la filière et cet agencement en quelque sorte physique des ateliers que nous adopterons par la suite sous l'appellation « processus classique » pour servir de base de comparaison avec les solutions innovantes devant résulter de la mise en œuvre des deux procédés, qu'ils soient combinés ou non dans le même établissement industriel.

Ce processus est figuré en solution n° 1 (fig. 1) : moyennement, il s'écoule une dizaine de jours après le jour J de l'abattage jusqu'à la commercialisation des produits portionnés prêts à la vente.

Par hypothèse, il est mis en œuvre une première réfrigération des carcasses

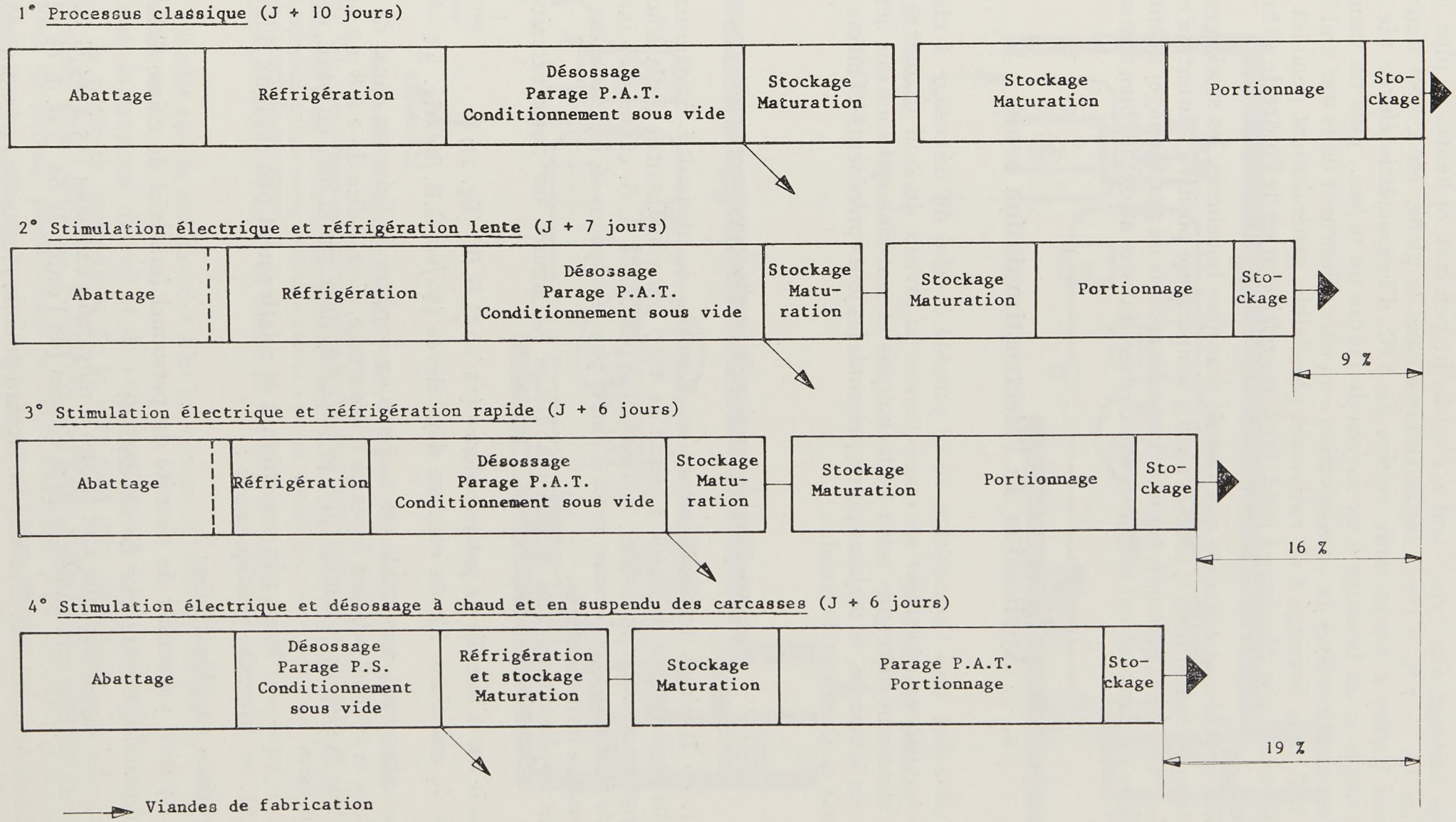


FIG. 1. — *Processus de travail des viandes fraîches de gros bovins : Étude de surface des locaux.*

dite de « ressuage » de type « lent », c'est-à-dire pendant 24 heures et plus (pour atteindre les + 7 °C à cœur réglementaires dans le globe, etc.), ceci dans des chambres froides à température voisine de 0 °C, d'hygrométrie élevée (de 90 à 95 p. 100), avec un brassage d'air moyen de l'ordre du m/sec., puis un transfert dans des chambres dites de « conservation » où elles séjournent dans une ambiance à 0 + 2 °C, avec brassage d'air nettement plus faible, généralement pendant 20 à 24 heures avant leur désossage à froid. D'où une réfrigération de l'ordre de 48 heures au total.

Par hypothèse et référence aux bonnes pratiques bouchères, les stockages suivants dits de « maturation », réalisés aussi à température positive, durent un minimum de 7 à 8 jours (y compris transport vers les ateliers de portionnage) avant que les viandes préparées soient effectivement commercialisées au détail, bien maturées.

2. — Application de l'électrostimulation seule

C'est-à-dire sans l'association de ce procédé à celui du désossage à chaud.

Pour analyser et évaluer ses conséquences au niveau du hall d'abattage, il faut évidemment distinguer selon les techniques et matériels qui sont mis actuellement sur le marché, en majeure partie présentés les jours précédents ici même ou à l'abattoir de Clermont-Ferrand.

1. — *Stimulation à « bas voltage »*

1.1. — Il s'agit essentiellement, en Europe, du dispositif à commande manuelle expérimenté en Suède à partir de 1977 par son Institut de la Viande, développé en abattoir industriel, mis au point par M.I.T A. B. et commercialisé à partir de juillet 1980 en France après d'autres pays européens où fonctionnent une cinquantaine d'installations.

Son implantation dans le hall d'abattage est figurée sur deux extraits de plans :

— dans le cas d'une file de petite cadence (15/20 G.B./h) (fig. 2);

— dans le cas d'une file de cadence déjà élevée (50/60 G.B./h) (fig. 3).

L'emplacement acceptable est toujours en bordure de l'aire ou canal de saignée, dans la zone et pendant le temps d'égouttage, c'est-à-dire de 2 à 8 mn après le début de la saignée et durant 30 à 40 secondes, donc sur la bête en peau, décolletée ou non.

Cet équipement léger, peu encombrant et relativement peu coûteux d'investissement, ne nécessite pratiquement :

— ni surface supplémentaire;

— ni dispositif particulier de sécurité du personnel (matériel de classe T.B.T.);

— ni appareillage complexe de sanitation;

— ni ouvrier supplémentaire aux faibles à moyennes cadences (15 à 35 G.B./h), mais un de plus aux cadences plus élevées (de l'ordre de 60 G.B./h et plus).

Il laisse la possibilité d'utiliser un trocard pour la récolte de sang alimentaire

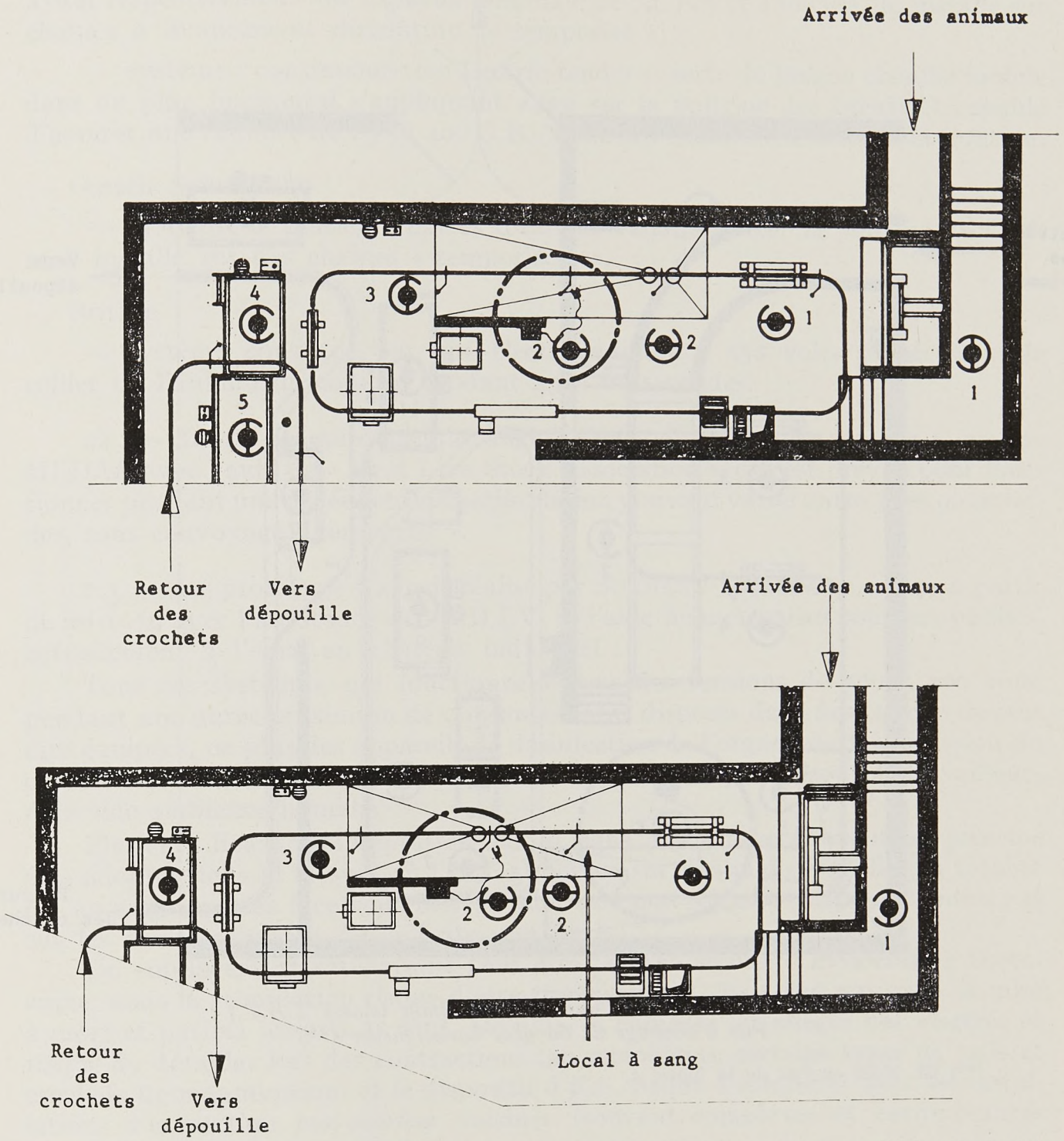


FIG. 2. — *Électrostimulation à bas voltage (classe T.B.T.) : File d'abattage de 15 gros bovins /heure* (*)

(*) *Note :* Les différents numéros portés sur les plans représentés dans les figures 2 à 5 indiquent la localisation du poste de travail correspondant aux diverses opérations unitaires de l'abattage.

L'affectation d'un même numéro à plusieurs postes signifie que c'est le même ouvrier qui assure les diverses opérations concernées.

Le lieu d'électrostimulation est localisé par un cercle en trait mixte.

et ne ralentit pas la cadence des files ou chaînes d'abattage de cadences faibles à moyennes.

Mais la propreté du rail et de l'élingue servant à la conduction du courant jusqu'à la patte de suspension de la bête doit être excellente.

D'autre part, cette E. S. précoce implique une rigidité également précoce qui, pour un certain pourcentage de bêtes abattues, peut entraîner une moindre facilité et productivité du travail, dans le cas où l'E.S. serait combinée avec le D.C.

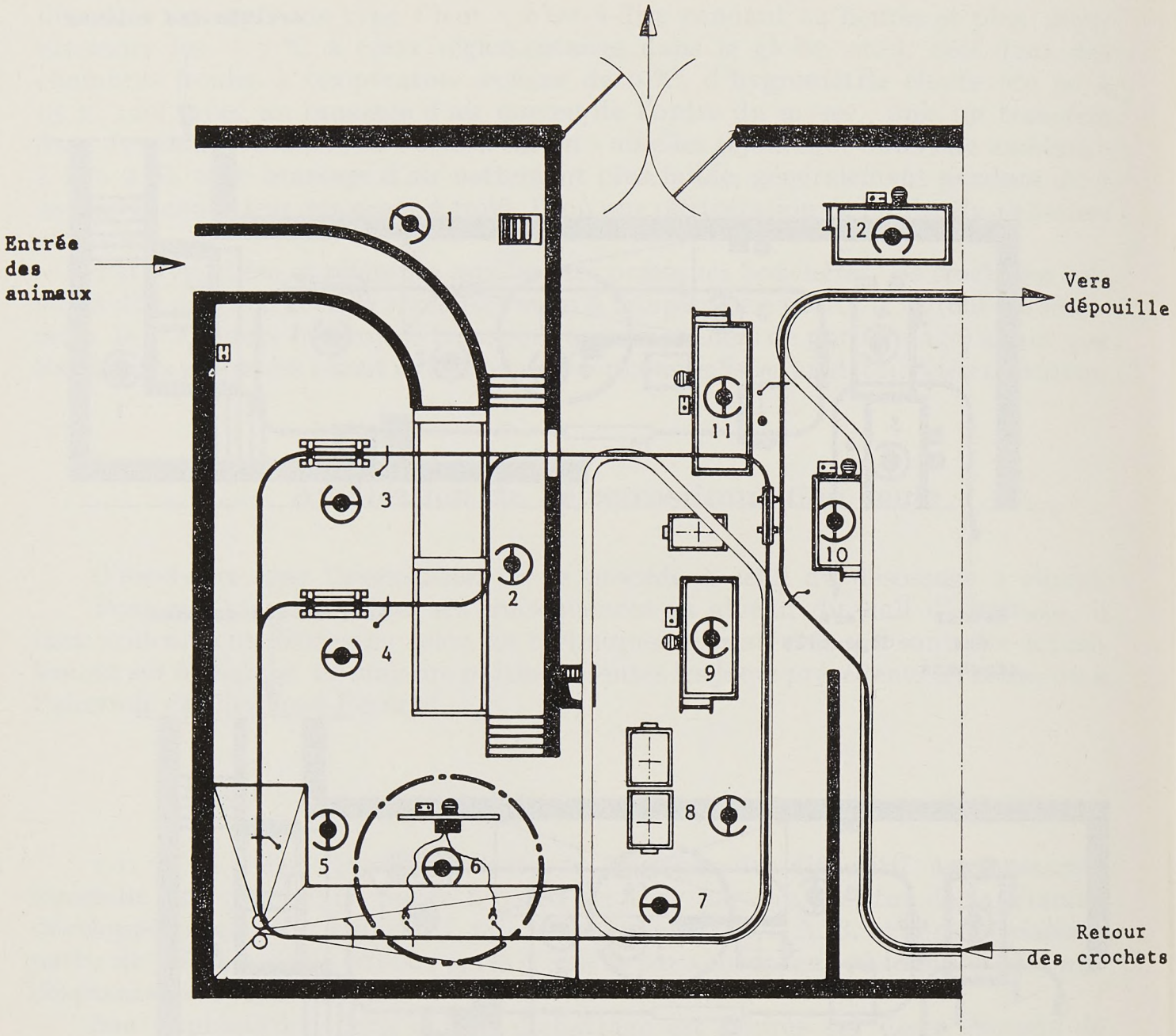


FIG. 3. — *Électrostimulation à bas voltage (classe T.B.T.) : File d'abattage de 60 gros bovins/heure* (**)

(**) Cf. Note en bas de la figure 2.

1.2. — Il s'agit aussi, pratiquement en Amérique, de l'appareillage mis au point et fabriqué par BRITTON : il doit avoir les mêmes avantages et inconvénients de principe.

2. — *Stimulation à « haut voltage »*

Il s'agit :

2.1. — des dispositifs américains développés très rapidement en 1978 aux U.S.A. à partir des résultats des travaux néo-zélandais et installés surtout à partir de 1979 dans plus d'une centaine d'abattoirs industriels plus ou moins importants par :

— Lefiell Co.

— Système tout automatique à 1, 2 ou 3 « barres » appliquées sur la poitrine,

ayant respectivement une capacité nominale de 70, 125 et 180 G.B. /h, installé sur chaînes à avancement discontinu (« temporisé »);

— système « continuous-trac Lectric-tender », sorte de longue chenille mobile dans un plan horizontal s'appliquant aussi sur la poitrine des bœufs et capable d'assurer une cadence de 150 à 400 G.B. /h sur des chaînes à avancement continu.

— Omeco Saint John

— système de plusieurs barres rotatives venant frotter la poitrine de l'animal, installé sur des chaînes « temporisées ».

— Britton

— système constitué par une électrode-sonde à 550 volts piquée dans le collier de l'animal immobilisé pendant 45 à 60 secondes.

22. — De l'unité automatique anglaise mise au point en 1979 par le groupe MEDAL avec l'aide du « Meat Live Stock Association », qui est prévue pour fonctionner pendant une durée totale de stimulation pouvant varier entre 30 et 90 secondes, sous convoyage « temporisé ».

2.3. — Du prototype français réalisé par SUCMANU-MARCHAND, conçu à partir de mi-1979 avec l'assistance de l'A.D.I.V. et l'aide financière des pouvoirs publics, actuellement à l'essai en abattoir industriel.

Tous ces systèmes, qui fonctionnent sous des tensions de 500 à 700 volts pendant une durée maximum de 2 minutes, sont disposés dans des cabines devant être équipées, en plus des appareils de désinfection de l'organe de transmission du courant au contact des chairs, de dispositifs très sûrs de protection des travailleurs dans une ambiance humide.

Plusieurs lieux d'implantation sur la ligne d'abattage peuvent en principe être adoptés dans la mesure où l'E.S. s'effectue sur l'animal dépouillé (en totalité ou, au moins, sur sa face ventrale), décolleté ou non selon les dispositifs, mais pas sur les demis résultant de la fente vertébrale.

Un emplacement satisfaisant peut être la zone suivant l'éviscération thoracique, mais la stimulation risque d'être trop tardive (plus de 30 mn après la mise à mort et parfois jusqu'à 45 mn). De plus, la carcasse est allégée des viscères et risque de dérailler lors des contractions (au moins avec certains types de rails et crochets de manutention) et le dispositif d'E.S. risque d'interférer avec les installations d'inspection *post-mortem* voisines (souvent complexes et encombrantes aux cadences moyennes à fortes).

Ces raisons font que nous préconisons, particulièrement dans les cas de construction neuve et de lignes quasi rectilignes, l'implantation de l'E.S. juste après la dépouille et avant l'éviscération abdominale. Le problème est d'éviter des émissions de fèces et d'urine et en conséquence de ne pas léser le sphincter anal avant la stimulation afin que sa contraction assure une fermeture parfaite du rectum. Une incision sur la ligne blanche doit être faite pour éviter aux viscères d'être trop comprimés dans la cavité abdominale au cours de la stimulation et des contractions qui s'ensuivent.

Les figures 4 et 5 illustrent cet agencement dans deux cas de cadence (25 et 60 G.B. /h) et deux types de matériels d'E.S.

Ces systèmes ne ralentissent pas non plus la cadence des files ou chaînes d'abattage dans la mesure où celle-ci est limitée à 35 /40 G.B. /h; au-delà, il y a vraisemblablement lieu de prévoir l'installation d'un autre poste. Mais, à toutes les

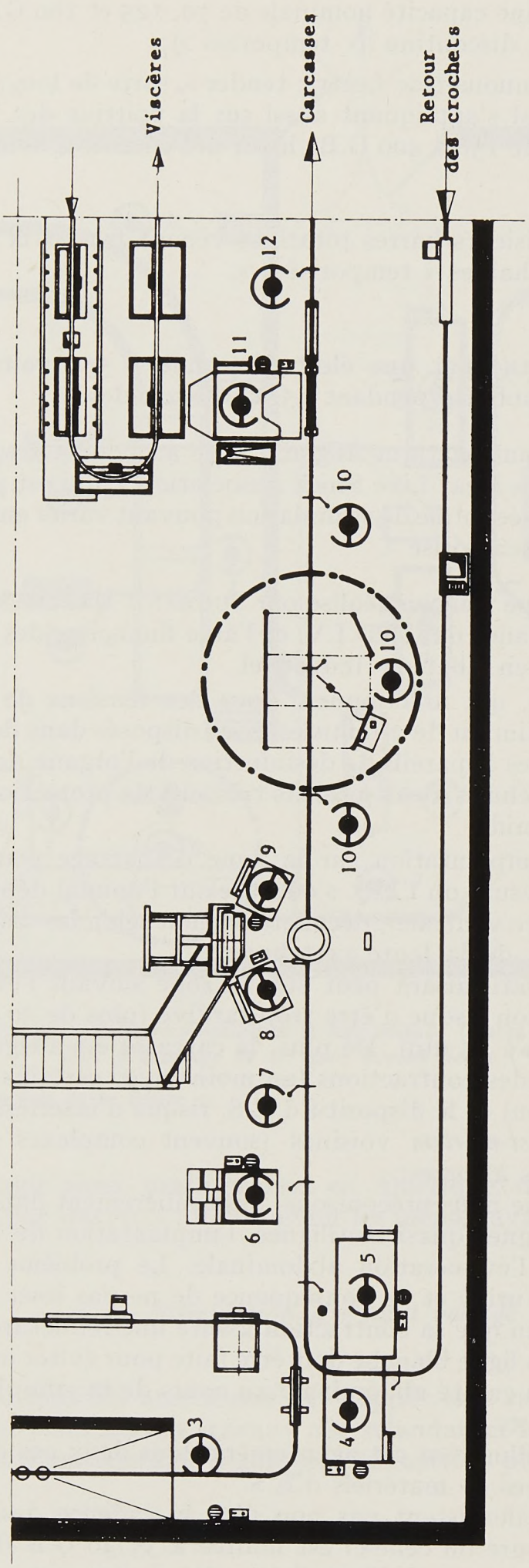


FIG. 4. — *Électrostimulation à haut voltage : File d'abattage de 25 gros bovins/heure (système manuel par électrode)* (**)

(**) Cf. Note en bas de la figure 2.

cadences pratiquées en France, le travail sur chaîne à avancement continu nous paraît poser problème au plan du convoyage.

Tous ces systèmes exigent une surface supplémentaire qui est estimée à environ 40 m² hors tout, soit 10 p. 100 de la surface du hall pour les capacités d'abattage indiquées ci-dessus, surface sans doute difficile à trouver dans le cas d'aménagement de lignes existantes à modifier sensiblement.

Enfin, les systèmes à commande manuelle nécessitent un ouvrier de plus, quelle que soit la cadence.

***

Quelles sont maintenant les conséquences au-delà du hall, sur la suite de la filière ?

On peut distinguer deux cas (cf. solutions n° 2 et n° 3 de la fig. 1) selon les conditions de la réfrigération qui, pour les bovins et dans la normalité française

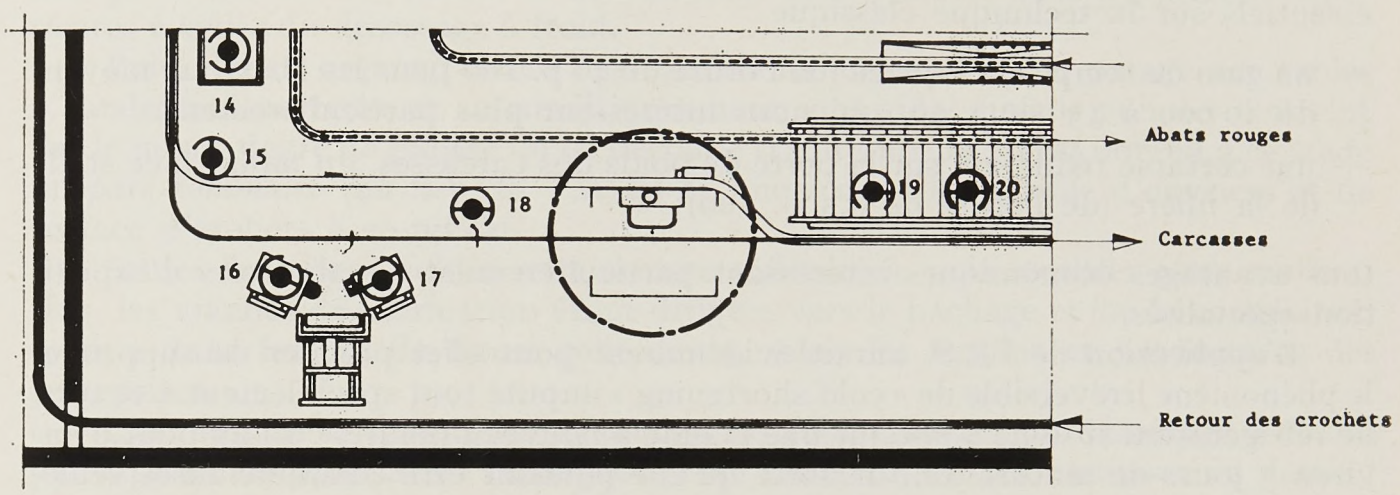


FIG. 5. — *Électrostimulation à haut voltage : Chaîne d'abattage de 60 gros bovins/heure (système automatique à barres)* (**)

(**) Cf. note en bas de la figure 2.

actuelle, doit s'effectuer au plus tard deux heures après leur étourdissement, mais est appliquée de préférence plus précocement dans les abattoirs de conception moderne, c'est-à-dire immédiatement après les dernières opérations et mesures dans le hall d'abattage et près de 3/4 d'heure *post-mortem*.

a) *Réfrigération lente*

Telle que précisé précédemment, encore très « classique » en particulier en Amérique du Nord, mais aussi en France.

L'E.S. modifierait la cinétique des phénomènes de maturation : en les accélérant de façon significative, la tendreté souhaitée serait atteinte plus rapidement et plus régulièrement, de sorte que le stockage de maturation pourrait être ramené à 5 jours environ et, par voie de conséquence, le volume total et le coût d'investissement des chambres froides correspondantes seraient réduits de l'ordre de 35 p. 100, pendant que la commercialisation pourrait valablement être avancée à J + 7. Le volume de l'ensemble des locaux frigorifiques de la filière (carcasses + produits) serait diminué de près de 20 p. 100 par rapport au processus classique dans ces conditions certes encore hypothétiques.

b) *Réfrigération rapide*

Dite « de choc » par certains ingénieurs-conseils, elle s'est développée en France la dernière décennie à partir de la Scandinavie : elle s'effectue en deux phases successives soit de façon discontinue dans des cellules statiques, soit, plus souvent, de façon continue, les carcasses de bovins étant alors convoyées automatiquement pendant 3 à 4 heures dans un tunnel où la température de soufflage de l'air est maintenue à des températures négatives (entre — 1 et — 3 °C, voire — 5 °C) et sa vitesse entre 2 et 3 m /seconde pour une humidité relative voisine de 95 p. 100, puis elles sont transférées toujours automatiquement dans une chambre de réfrigération complémentaire au voisinage de 0 °C et en air peu brassé.

La puissance frigorifique requise doit être de 50 à 100 p. 100 supérieure à celle des chambres classiques.

Mais, outre la stabilisation précoce et rapide du développement microbien souhaitée par les hygiénistes, cette technique de réfrigération a pour avantages essentiels sur la technique classique :

— un gain de temps et de place (de l'ordre de 40 p. 100 pour les abattoirs moyens de 10 000 à 15 000 t /an, qui nous intéressent plus particulièrement);

— une certaine réduction sur la perte de poids des carcasses, du moins à ce stade de la filière (de l'ordre de 0,5 p. 100);

tous avantages économiques intéressant particulièrement les abattoirs d'expédition spécialisés.

L'application de l'E.S. aurait évidemment pour effet premier de supprimer le phénomène irréversible de « cold shortening » imputé tout spécialement à ce type de réfrigération et donc d'obtenir une viande à nouveau tendre, ceci au bout d'environ 5 jours de maturation, de sorte qu'elle pourrait être commercialisée seulement 6 jours après l'abattage. Ainsi, les temps de réfrigération et de stockage des carcasses puis des produits élaborés tendraient à être réduits de près de moitié.

Le volume total des locaux frigorifiques pris en considération serait, quant à lui, diminué de plus de 35 p. 100.

Bien entendu, dans les deux cas examinés d'utilisation de l'E.S., l'organisation du travail et la préparation des viandes fraîches en ateliers industriels ainsi que les locaux correspondants restent par hypothèse identiques à la solution de base.

3. — Application du désossage à chaud associé à l'E.S.

Dans cette hypothèse véritablement « révolutionnaire » (à laquelle correspond la solution n° 4 de la fig. 1), toutes les conditions et tous les locaux de réfrigération des carcasses qui ont été indiqués précédemment n'existent plus, les opérations conventionnelles de ressuage et de conservation étant supprimées.

Ces carcasses préalablement stimulées sont introduites immédiatement et directement dans l'atelier *contigu* de découpage-désossage, suspendues aux rails de manutention et travaillées avant l'installation de la rigidité, donc à l'état pantelant pour faciliter le travail ultérieur de mise en morceaux, muscles et pièces.

Ceci implique de le réaliser *en continuité de l'abattage*, à la cadence même des opérations.

Cette solidarité et ce synchronisme obligatoires constituent une contrainte

d'importance, surtout lorsque l'on atteint les cadences « moyennes » de 30 à 40 G.B. /h aujourd'hui pratiquées en France dans les abattoirs industriels modernes, qui correspondent au travail annuel d'environ 50 000 gros bovins, soit 15 000 tonnes en poids-carcasse. C'est le cas de figure étudié : il faut alors doubler la capacité de travail d'ateliers de découpe actuellement considérés comme de grand format, en supposant par hypothèse que l'on multiplie très grandement, si ce n'est d'autant, le nombre des couteaux et le volume de l'atelier faute d'une véritable technologie industrielle à ce stade. (Celle-cî implique mécanîsation et automatisation de l'ensemble, si ce n'est robotisation, qui sont certes envisagées, mais n'en sont qu'au tout début de la recherche d'amont relative aux systèmes de coupe).

Pour des raisons de meilleures hygiène et productivité du travail, l'option dé *désossage en suspendu* et en quelque sorte par gravité, déjà pratiquée dans certains pays anglo-saxons et au Danemark, est préconisée. Elle devrait amener à une réduction de l'ordre du quart des personnels de désosse, d'ailleurs moins qualifiés, pour une surface au sol et une hauteur sous plafond utile légèrement supérieures à celles du désossage à froid.

Puis, dans l'organigramme des opérations, on revient au parage sur tables à bande transporteuse et au conditionnement sous vide, mais le premier n'atteint pas le même degré de parage qu'en découpe traditionnelle : il est obtenu à ce stade un *paré sommaire* (au lieu du P.A.T.) et une économie sensible d'ouvriers et de surface d'ateliers à ce niveau.

Seules les pièces nobles sont ensuite réfrigérées, puis stockées pour maturation, les viandes de fabrication étant dirigées vers le hachage et /ou la congélation comme dans les cas de figure précédents, selon les besoins et productions des entreprises.

C'est là que se situent les avantages les plus évidents du désossage à chaud par rapport au processus « classique » avec réfrigération des carcasses ou demis :

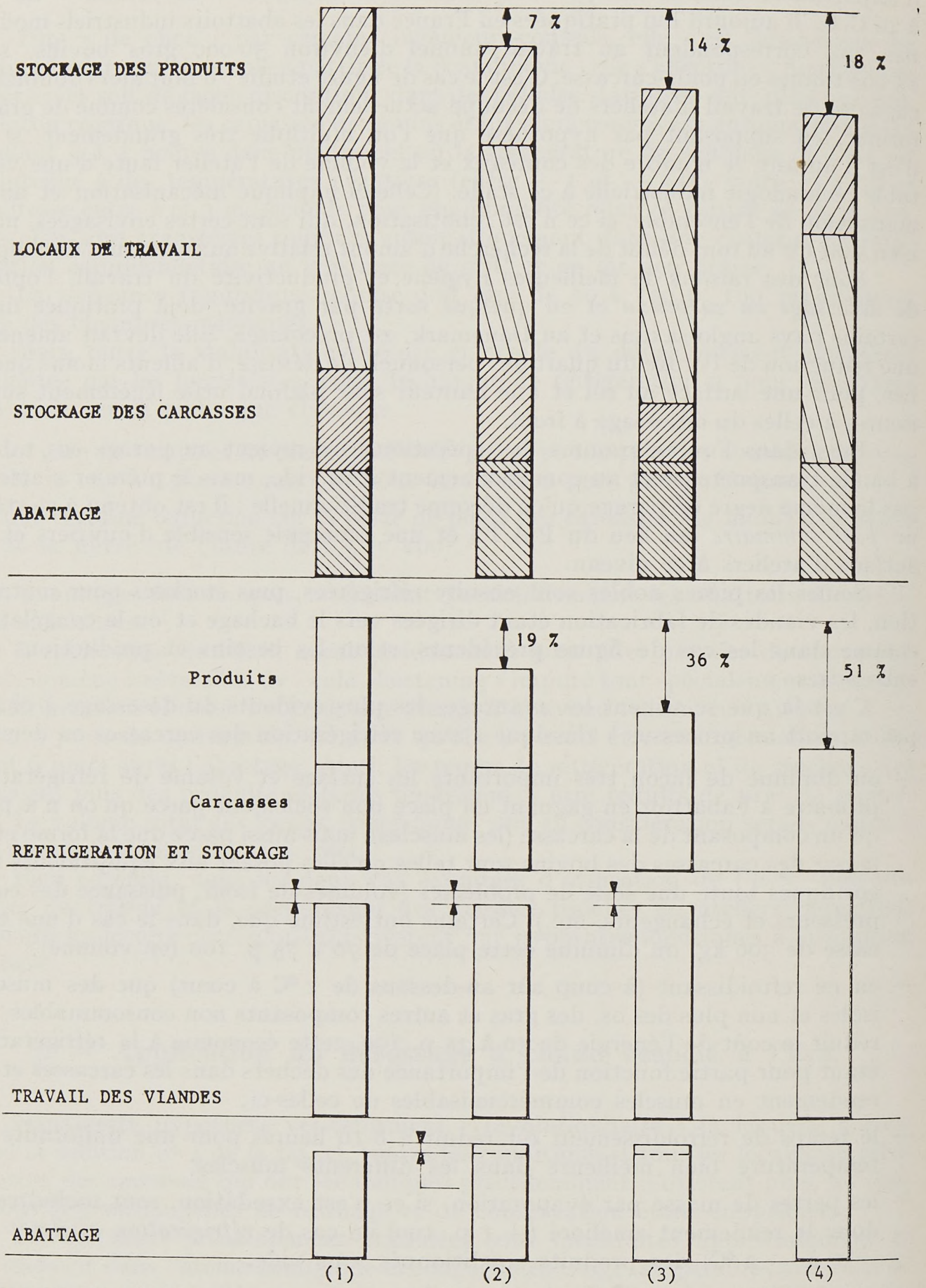
— on diminue de façon très importante les surface et volume de réfrigération primaire à l'abattoir en gagnant en place non seulement parce qu'on n'a plus qu'un composant de la carcasse (les muscles), mais aussi parce que la forme et la masse des carcasses des bovins sont telles qu'elles posent aux équipements frigorifiques toute une série de problèmes (volumes de froid, puissance des compresseurs et échangeurs, etc.). Certains ont estimé que, dans le cas d'une carcasse de 300 kg, on diminue cette place de 70 à 75 p. 100 (en volume);

— en ne refroidissant (à coup sûr au-dessous de 7 °C à cœur) que des muscles isolés et non plus des os, des gras et autres composants non consommables, on réduit le coût de l'énergie de 30 à 35 p. 100, cette économie à la réfrigération étant pour partie fonction de l'importance des déchets dans les carcasses et du rendement en muscles commercialisables de celles-ci;

— le temps de refroidissement est réduit à 8-10 heures pour une uniformité de température bien meilleure dans les différents muscles;

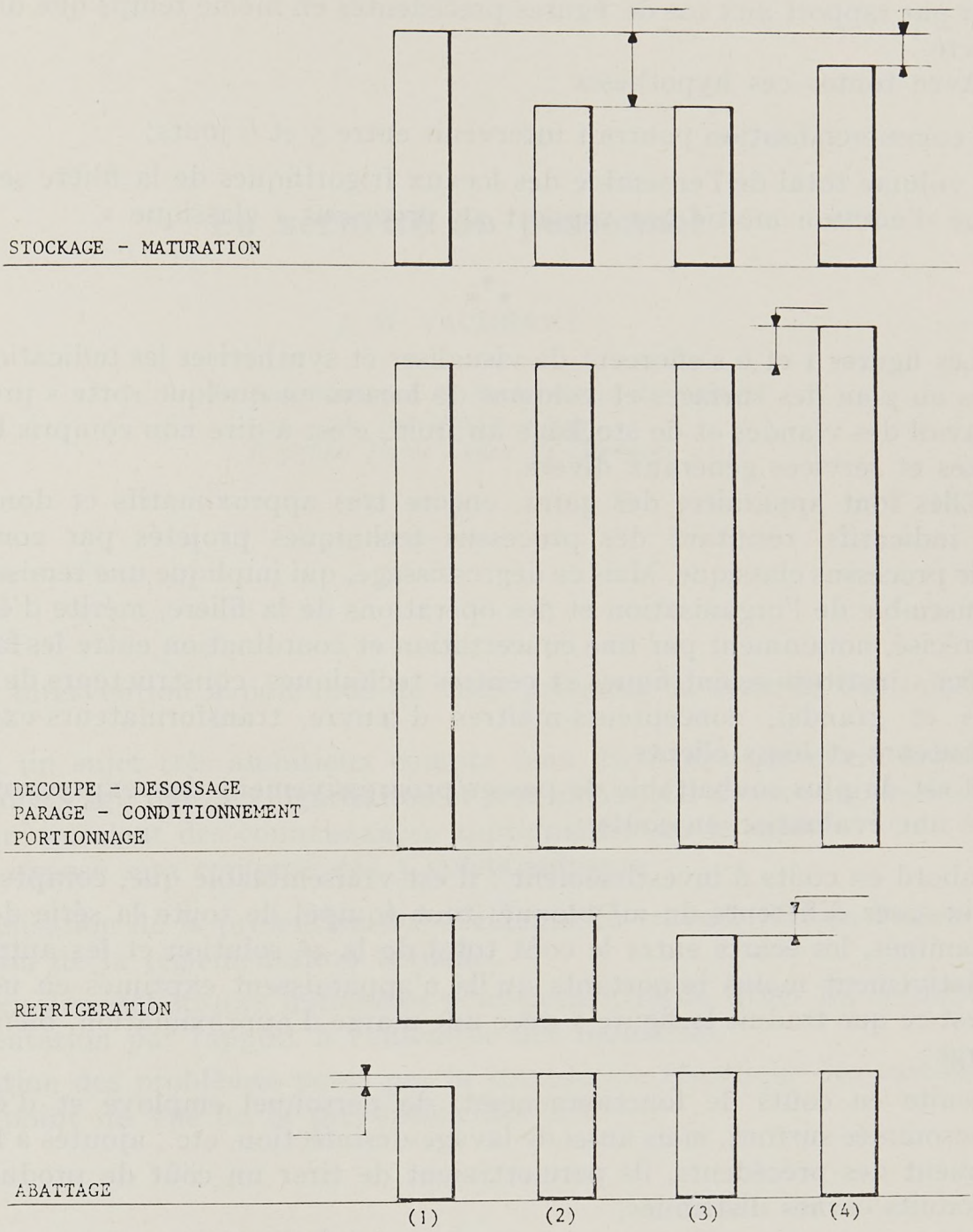
— les pertes de masse par évaporation, si ce n'est exsudation, sont moindres et donc le rendement amélioré (+ 1 p. 100) en cas de *réfrigération accélérée* (de — 1 à — 2 °C) des produits conditionnés sous vide.

Pour l'évaluation des surfaces et volumes des chambres de réfrigération et de stockage maturation (d'un jour) — cf. figures 1 et 6 —, nous avons supposé que ces produits étaient disposés sur chariots « ouverts » roulant au sol du type caddytainer, donc dans des chambres basses, ceci afin d'une part de permettre une circulation d'air froid suffisante, d'autre part de faciliter, si ce n'est automatiser,



(1) Processus classique (J + 10 jours)
(2) Stimulation électrique et réfrigération lente (J + 7 jours)
(3) Stimulation électrique et réfrigération rapide (J + 6 jours)
(4) Stimulation électrique et désossage à chaud et en suspendu des carcasses (J + 6 jours)

FIG. 6. — *Processus de travail des viandes fraîches de gros bovins : Étude de volume des locaux*



(1) Processus classique (J + 10 jours)
(2) Stimulation électrique et réfrigération lente (J + 7 jours)
(3) Stimulation électrique et réfrigération rapide (J + 6 jours)
(4) Stimulation électrique et désossage à chaud et en suspendu des carcasses (J + 6 jours)

FIG. 7. — *Processus de travail des viandes fraîches de gros bovins : Étude comparative des investissements*

les manutentions jusqu'à la sortie de l'établissement et, au-delà, les transports.

Mais d'autres solutions techniques peuvent être envisagées : carrousels convoyés, containers gerbés, etc., qui dépendent de considérations économiques (coûts d'investissement et de fonctionnement) et de contraintes d'exploitation.

Enfin, en bout de la partie de filière examinée et dans l'étude de cas exposée, le portionnage est précédé d'un parage complémentaire pour aboutir à un P.A.T. de bonne présentation à la vente, ce qui entraîne une augmentation sensible de cet

atelier par rapport aux cas de figures précédentes en même temps que de la main-d'œuvre.

Avec toutes ces hypothèses :

— la commercialisation pourrait intervenir entre 5 et 6 jours;

— le volume total de l'ensemble des locaux frigorifiques de la filière serait diminué d'environ moitié par rapport au processus « classique ».

***

Les figures 1 et 6 s'efforcent de visualiser et synthétiser les indications précédentes au plan des surfaces et volumes de locaux en quelque sorte « productifs » de travail des viandes et de stockage au froid, c'est-à-dire non compris les locaux annexes et services généraux divers.

Elles font apparaître des gains, encore très approximatifs et donc simplement indicatifs, résultant des processus techniques projetés par comparaison avec le processus classique. Mais ce dégrossissage, qui implique une remise en cause de l'ensemble de l'organisation et des opérations de la filière, mérite d'être peu à peu précisé, notamment par une concertation et coordination entre les familles en présence : instituts scientifiques et centres techniques, constructeurs de matériels (petits et grands), concepteurs-maîtres d'œuvre, transformateurs-exploitants, distributeurs et leurs clients.

Il est de plus souhaitable de passer progressivement des expressions physiques à une évaluation en coûts :

— d'abord en coûts d'investissement : il est vraisemblable que, compte tenu des prix assez différents du m^2 bloqué (tout équipé) de toute la série des locaux examinés, les écarts entre le coût total de la 4e solution et les autres seront relativement moins importants qu'ils n'apparaissent exprimés en m^2 ou m^3; c'est ce que traduit la figure 7 avec une marge d'approximation, bien sûr, très large;

— ensuite en coûts de fonctionnement, de personnel employé et d'électricité consommée surtout, mais aussi de lavage-désinfection, etc.; ajoutés à l'amortissement des précédents, ils permettraient de tirer un coût de production des produits carnés distribués;

— mais aussi en coût global, c'est-à-dire le coût initial (prix du terrain, dépenses de construction, honoraires) augmenté de la somme des valeurs actualisées des coûts qui lui seront imputables pendant toute la période d'exploitation des ouvrages (maintenance, grosses réparations, consommations, etc.) et ce en fonction des variations probables des coûts de chacun des facteurs de production, de l'énergie tout particulièrement.

Le C.T.G.R.E.F. est disposé à collaborer à cette étude technico-économique sur la base de résultats de la recherche technologique mieux précisés ou plus affirmés.

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La sécurité du personnel

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Mon intervention a pour but de vous présenter la sécurité des salariés en France.

C'est un sujet très ambitieux compte tenu du temps qui m'est imparti. Il ne peut donc s'agir que de vulgarisation et je m'excuse par avance auprès des participants qui auraient des connaissances approfondies sur le sujet.

Mon exposé sera composé des 3 volets suivants :

1. L'organisation de la présention des accidents du travail (AT) en France et la situation de la réglementation actuelle.
2. Examen des statistiques nationales pour permettre de situer les industries de l'alimentation par rapport à l'ensemble des industries.
3. Évocation des problèmes posés par la stimulation électrique des carcasses du strict point de vue de la prévention des A.T.

1. — Organisation de la prévention

Généralités

Depuis 1946 :

— l'assurance « accident » est obligatoire;

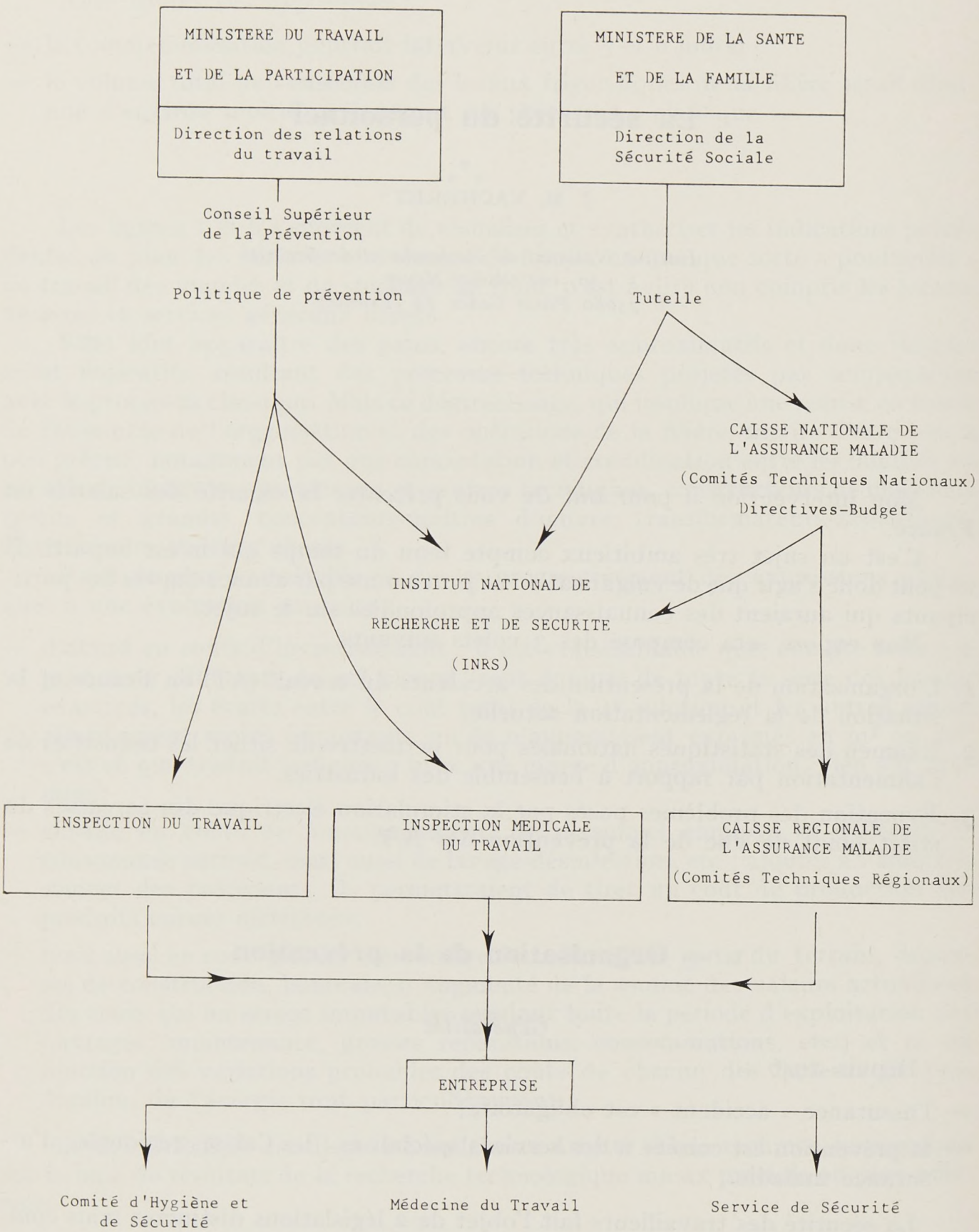
— la prévention est confiée à des services spécialisés : les Caisses régionales d'assurance maladie.

La sécurité des travailleurs fait l'objet de 2 législations distinctes mais complémentaires :

1) Législation d'ordre public imposée par le Code du travail, élaborée par la Direction des relations du travail (Ministère du travail).
2) Législation préventive et réparatoire issue du Code de la Sécurité sociale élaborée par la direction de la Sécurité Sociale (Ministère de la Santé).

TABLEAU I

Organisation de la prévention



L'organisation de la prévention est représentée schématiquement dans le tableau I.

La direction des relations du travail établit les textes relatifs à l'hygiène et à la sécurité des travailleurs et à la médecine du travail (Arrêtés et décrets qui peuvent être intégrés au Code du Travail).

Tous ces textes sont établis après avis du Conseil supérieur de la prévention au sein duquel les partenaires sociaux sont représentés.

Les inspecteurs du travail sont chargés de veiller à l'application de ces textes.

La direction de la Sécurité Sociale exerce sa tutelle sur les organismes de Sécurité Sociale qui, en matière de prévention, sont les CNAM (Caisse Nationale de l'Assurance Maladie), les 16 C.R.A.M. (Caisses Régionales d'Assurance Maladie).

La C.N.A.M. est assistée des C.T.N. (Comités Techniques Nationaux) et les C.R.A.M. des C.T.R. (Comités Techniques Régionaux) comités qui sont des organismes paritaires constitués par branche professionnelle.

Par voie de D.G. (Dispositions Générales) chaque Caisse régionale peut soumettre l'ensemble des employeurs de sa circonscription à des mesures particulières de prévention. Elles peuvent être étendues à l'ensemble du territoire sur proposition des C.T.N. et la C.N.A.M.; elles font alors l'objet d'un arrêté qui les intègre au Code de la Sécurité Sociale.

Ces D.G. découlent de la gestion du risque réel constaté en entreprise, elles vont au-delà du minimum réglementaire.

Les organismes privés intervenant dans la prévention sont : l'Office professionnel de Prévention du Bâtiment et Travaux publics (O.P.P.B.T.P.) et l'Institut national de Recherche et de Sécurité (I.N.R.S.).

L'Institut National de Recherche et de Sécurité (I.N.R.S.)

Les caractéristiques générales de cet organisme sont indiquées dans le tableau 2.

L'I.N.R.S. est un organisme de droit privé, à conseil d'administration paritaire, placé sous la tutelle de la C.N.A.M. et des pouvoirs publics.

En quelque sorte c'est le Bureau d'étude de la C.N.A.M. et des C.R.A.M. mais les bénéficiaires de ses travaux sont multiples puisqu'il développe ses activités à caractère de service public dans trois directions :

— l'assistance technique;

— la formation et l'information;

— les études et recherches.

L'organisation de l'I.N.R.S. est présentée dans le tableau 3. L'I.N.R.S. comprend au total 487 agents répartis dans les différents services du centre de Paris (169 agents) et de Nancy (318 agents).

Les études de l'I.N.R.S. concernent des problèmes qui présentent un intérêt pour l'ensemble de l'industrie. Le tableau 4 en précise les différentes natures et les divers objectifs.

Réglementation actuelle

La législation mise en place en 1946 a permis de faire chuter d'une façon sensible le nombre des A.T. en France.

Cependant une certaine stabilité des A.T. est apparue vers 1970. Cette constatation a conduit le législateur à élaborer la *loi du 6 décembre 1976* qui vise à renforcer la sécurité du travail et à améliorer certaines modalités de la réparation des dommages subis du fait du travail.

Elle prévoit notamment :

— l'obligation de former les salariés à la sécurité;

TABLEAU 2

Caractéristiques générales de l'I.N.R.S.

Origine : I.N.R.S. 1968 . . . . . { — 1947 : Création de l'Institut National de Sécurité et de la Caisse Nationale de l'Assurance Maladie. — 1968 : Centre d'Études et de Recherches.

Mission : Contribuer sur le plan technique à la prévention des accidents du travail et des maladies professionnelles.

— Études . . . . . { — procéder aux études et aux recherches.

— Formation Information { — recueillir, élaborer, diffuser la documentation; — programmes et méthodes de formation.

— Concours technique . . { — apporter un concours technique :
⊕ aux ministères du travail et de la santé;
⊕ Caisse Nationale d'Assurance Maladie et Caisse Régionale d'Assurance Maladie;
⊕ Comité d'hygiène et de sécurité et Comités Techniques Nationaux.
— coopérer sur le plan international :
⊕ Bureau international du travail;
⊕ Association internationale de Sécurité Sociale.

Statuts : *Tutelle* du Ministère chargé de la Sécurité Sociale.

Politique de prévention : Ministère du travail.

Directives : Caisse Nationale d'Assurance Maladie.

Conseil d'administration paritaire { 9 représentants patronaux, 9 représentants salariés assistants de droit.

Financement : Fonds national de prévention.

TABLEAU 3

Organisation de l'I.N.R.S.

Conseil d'Administration

Président

Direction Générale

Centre de Paris	Centre de Nancy
— Administration.	— Chimie, Toxicologie, Pathologie professionnelle.
— Formation	— Ergonomie, Physiologie, Psychologie.
— Sous Direction Technique.	— Machines dangereuses.
— Service Médical.	— Physique.
— Information.	— Moyens généraux.
— Documentation.	

TABLEAU 4

Les études de l'I.N.R.S.

Les équipements de protection (Protection collective et protection individuelle)	⊕ Guider le choix des utilisateurs. ⊕ Orienter les constructeurs et fabricants. ⊕ Normalisation et réglementation.
Les matériels industriels	⊕ Informer les utilisateurs et fabricants. ⊕ Méthodes d'essais. ⊕ Améliorer la conception. Ex. : machines à bois; machines outils (meules, presses); matériels de chantiers; matériels de manutentions.
Études des risques en situation industrielle	⊕ Phénomène accident. ⊕ Les conditions de travail. ⊕ La charge physique et la charge mentale. ⊕ Enquêtes épidémiologiques.
Produits chimiques utilisés dans l'industrie	⊕ Enquêtes épidémiologiques. ⊕ Formation des substances toxiques. ⊕ Toxicité spécifique des composants. ⊕ Lutte technique contre les substances toxiques.

— la limitation progressive du travail par équipes, des cadences et des rythmes, s'ils affectent l'hygiène et la sécurité;

— l'interdiction de travail au rendement dans les cas des travaux pénibles ou insalubres;

— l'obligation d'informer des organismes agréés avant de mettre certains produits sur le marché; ces organismes devront apprécier les risques d'emploi de ces produits dont la fabrication, la vente ou l'importation pourront être interdites;

— l'obligation de soumettre certaines machines à un contrôle préalable à la première vente, d'autres machines devant être seulement conformes à un « cahier de charges de sécurité; »

— certaines règles de construction et d'aménagement relatives à la sécurité pour les bâtiments à usage industriel, commercial ou agricole;

— l'obligation, dans les chantiers, pour le maître d'ouvrage de coordonner les mesures de sécurité prises, jusqu'alors, en ordre dispersé par les entrepreneurs.

En 1979 et 1980 plusieurs Décrets ont été adoptés en application de la loi du 6 décembre 1976 :

* Décret 79-228 du 20-03-1979 :
Comité d'hygiène et de sécurité : Formation à la sécurité.

* Décret 79-230 du 20-03-1979 :
Substandes et préparations dangereuses pour les travailleurs.

* Décret 79-230 du 20-03-1979 :
Organisation et fonctionnement des services médicaux du travail.

* Décret 79-229 du 20-03-1979 :

Machines dangereuses — Procédures destinées à la mise en œuvre des règles d'hygiène et de sécurité auxquelles doivent satisfaire les appareils, machines, éléments de machines, les protecteurs de machines, les dispositifs, équipements et produits de protection, contrôle préalable pour les machines les plus dangereuses, autocertification pour les autres.

Trois textes complémentaires de ce dernier décret sont parus le 15 juillet 1980 :

* Décret 80-542 déterminant une première liste des matériels auxquels s'appliquent les dispositions du 1er alinéa de l'article L. 233-5 du Code du travail, selon lequel :

« Il est interdit d'exposer, de mettre en vente, de vendre, d'importer ou de louer, de céder à quelque titre que ce soit ou d'utiliser :

a) des appareils, machines ou éléments de machines qui ne sont pas construits, disposés, protégés ou commandés dans des conditions assurant la sécurité et l'hygiène des travailleurs;

b) des protecteurs de machines ainsi que des dispositifs, équipements ou produits de protection qui ne sont pas de nature à garantir les travailleurs contre les dangers de tous ordres auxquels ils sont exposés. »

La liste des matériels visés par le décret 80-542 comprend notamment : « les machines agricoles et matériels pour les industries agro-alimentaires, autres que les machines agricoles mobiles ».

* Décret 80-543 fixant les règles d'hygiène et de sécurité applicables aux machines qui ne sont pas les plus dangereuses.
Les machines utilisées dans les industries alimentaires sont concernées par ce décret.

* Décret 80-544 fixant des règles particulières d'hygiène et de sécurité applicables à d'autres machines que celles de l'industrie alimentaire, notamment les machines-outils.

Compte tenu de l'importance et de la complexité de ces décrets l'I.N.R.S. a publié une brochure explicative destinée aux utilisateurs, constructeurs, importateurs (*).

Dans le domaine qui nous préoccupe aujourd'hui la grande innovation consiste à obliger les constructeurs... d'établir une attestation de conformité (Procédure dite « d'auto-certification ») par laquelle il s'engage à ce que leur matériel réponde aux exigences des décrets.

Jusqu'à présent les textes relatifs à la sécurité des travailleurs ne s'adressaient qu'aux employeurs utilisateurs; c'est pourquoi les constructeurs travaillent actuellement au S.Y.G.E.C.A.M. (Syndicat des Matériels de l'Alimentation) afin de proposer à l'U.N.M. (Union de Normalisation de la Mécanique) des normes de sécurité pour les machines.

(*) Intégration de la sécurité dans la conception des machines et appareils (Les décrets du 15 juillet 1980). Édition I.N.R.S. N° 623 — Code commande 11562.

2. — Statistiques concernant les accidents du travail

Les statistiques nationales sont publiées chaque année par la C.N.A.M. et les dernières en date sont celles de 1978. Elles ne concernent que les industries relevant du régime général de la Sécurité Sociale. (Les établissements relevant du régime agricole sont donc exclus de ces statistiques).

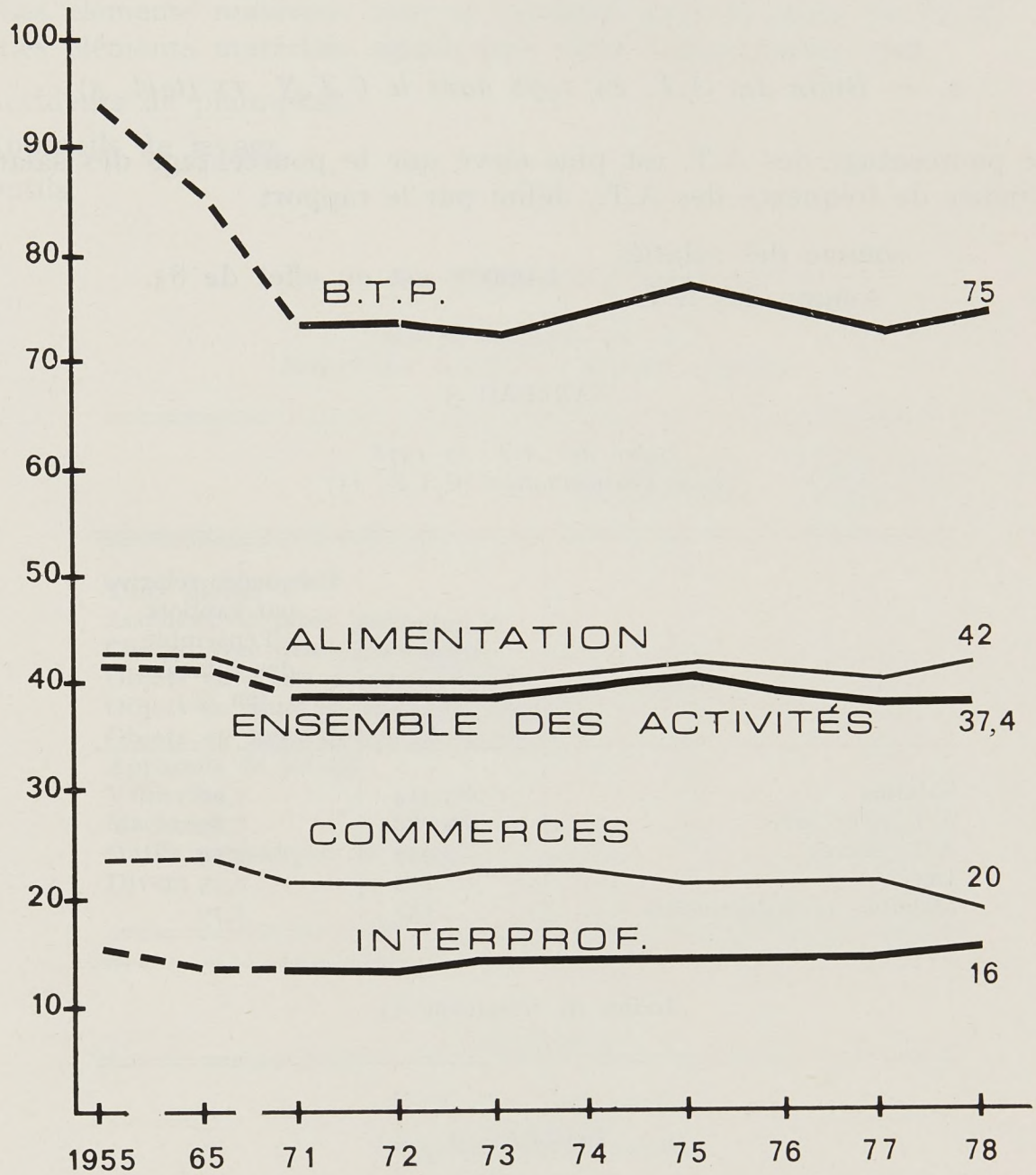


FIG. 1. — *Variations du taux de fréquence des A.T. avec arrêt de 1955 à 1978*

Les industries du régime général sont classées en 16 C.T.N. (Comités techniques nationaux) suivant leur secteur d'activité.

Ex. : C.T.N. 01 Métallurgies;
C.T.N. 02 B.T.P.;
C.T.N. 11 Industries et commerce de l'alimentation.

Nous nous bornerons à dégager les éléments statistiques significatifs du C.T.N. 11.

1) *Variation du taux de fréquence des A.T. avec arrêt de 1955 à 1978 (fig. 1)*

Le taux de fréquence est $\frac{\text{somme des A.T.}}{\text{somme des heures travaillées}} \times 10^6$.

Le C.T.N. 11 est représentatif de l'ensemble des industries mais depuis 3 ans on peut constater une augmentation de ce taux qui est de 42 pour l'alimentation contre 37,4 dans l'ensemble des industries.

2. — *Bilan des A.T. en 1978 dans le C.T.N. 11 (tabl. 5)*

Le pourcentage des A.T. est plus élevé que le pourcentage des salariés. L'indice de fréquence des A.T., défini par le rapport :

$\frac{\text{somme des salariés}}{\text{somme des A.T.}} \times 1\,000$ y est en effet de 83.

TABLEAU 5

Bilan des A.T. en 1978 dans l'alimentation (C.T.N. 11)

	Nombre total	Fréquence relative par rapport à l'ensemble des salariés (en %)
Salariés	1 083 314	7,90
A.T. avec arrêt	89 789	8,85
A.T. graves	7 957	7,54
Décès	112	7,15
Maladies professionnelles	152	3,79

Indice de fréquence 83

TABLEAU 6

Indices de fréquence des A.T. (pour 1 000 salariés)

Ensemble de l'alimentation	83
Commerce en gros des boissons	91
Travail des farines	92
Commerce des viandes	124
Conserveries	136
Charcuterie-conserveries avec abattage	163
Abattage de bétail	257

Le C.T.N. 11 étant décomposé en groupes et sous-groupes, il est possible de dégager les secteurs les plus dangereux (tabl. 6).

L'indice de fréquence de 257 dans l'abattage du bétail se passe de commentaire puisqu'il indique qu'un ouvrier sur quatre se blesse tous les ans.

3. — *Répartition des A.T. suivant les éléments matériels (tabl. 7)*

Les éléments matériels sont en quelque sorte la cause de l'A.T.

Les éléments matériels significatifs dans l'alimentation sont :

— Accidents de plain-pied.
— Appareils de levage.
— Outils.

TABLEAU 7

Taux de fréquence des A.T.
Répartition suivant les élements matériels

	Ensemble des salariés	Alimentation (C.T.N. 11)
Taux global	37,4	42
Accident de plain pied	7	8
Chutes avec dénivellation	4,4	3,9
Objets en cours de manipulation.	10,4	10,4
Objets en cours de transport manuel	1,7	2,2
Objets en mouvement accidentel.	3,4	1,8
Appareils de levage	1,5	2,9
Véhicules	1,1	1,2
Machines	3,2	2,7
Outils mécaniques et à main	2,9	6,2
Divers	1	1,2

TABLEAU 8

C.T.N. 11 Alimentation
Maladies professionnelles
Année 1978

Nombre total de maladies : 152

dont

Brucelloses	95
Affections respiratoires	12
Dermatophities d'origine animale	5
Leptospiroses	4
Affections tuberculeuses	3

4. — *Maladies professionnelles du C.T.N. 11 (tabl. 8)*

Pour le C.T.N. de l'alimentation il y a moins de maladies professionnelles que pour le reste des activités mais, en 1978, 95 cas de Brucellose sont encore à déplorer.

5. — *Statistiques concernant spécialement le commerce des viandes (tabl. 9)*

Les outils individuels à main sont la cause de 38,56 p. 100 des A.T. C'est une conséquence directe de la technique actuelle du travail de la viande.

TABLEAU 9

Groupe 11-04 : Commerce des viandes

	Nombre	Fréquence relative dans le C.T.N. 11 en p. 100
Salariés	134 256	12,39
A.T. avec arrêt	16 627	18,51
A.T. graves	1 269	15,95

Indice de fréquence 124

Éléments matériels des accidents du travail	Nombre	Fréquence des accidents du travail du groupe 11-04
Outils individuels à main. . .	6 413	38,56
Manutention manuelle	3 469	20,86
Chutes de plain-pied	1 967	11,83
Chutes de hauteur	701	4,21
Scies	482	2,89

3. — Problèmes posés par la stimulation électrique

Les appareils de stimulation électrique devront répondre à compter du 1er avril 1981 aux exigences des décrets du 15 juillet 1980.

Compte tenu de la nouveauté de cette technique, une Caisse régionale a sollicité l'avis de l'I.N.R.S. pour définir les exigences de prévention à mettre en œuvre pour assurer la sécurité des travailleurs pendant les opérations de stimulation électrique.

D'une manière générale notre point de vue est le suivant :

1) A chaque fois que cela est possible, notamment lors de l'implantation de nouveaux établissements, la véritable sécurité consiste à éloigner le personnel de la zone dangereuse en surélevant la cabine « tunnel » au-dessus du sol de l'abattoir.

2) En cas d'impossibilité, notamment dans les abattoirs en service, la mise hors de portée peut s'effectuer par obstacle.
La cabine de stimulation doit donc être munie de portes se fermant automatiquement après l'introduction de l'animal.

La stimulation électrique sera asservie à la fermeture effective des portes ainsi qu'à l'occultation d'une cellule (ou d'un autre dispositif équivalent) chargée de s'assurer que la carcasse est effectivement à sa place.

Cependant un risque subsiste pour une personne entrant dans la cabine en même temps que la carcasse, c'est pourquoi le plancher de la cabine sera installé sur des dispositifs de sécurité (cellules, contacts électriques, électromagnétiques) interdisant la stimulation si une personne circule dans la cabine.

Pendant les opérations de nettoyage et d'entretien qui sont très souvent source d'A.T. puisqu'elles nécessitent l'introduction de personnel dans la zone dangereuse, l'installation devra être consignée. Pour s'assurer de la bonne consignation un système de « clés prisonnières » tel que celui installé sur les postes de transformation sera la meilleure solution.

Il n'en demeure pas moins vrai que, du strict point de vue de la prévention, la stimulation H.T. est à déconseiller compte tenu de l'ambiance particulièrement humide qui règne dans les abattoirs.

Cas de la stimulation électrique en basse tension

Dans les opérations de stimulation B.T. où une opération manuelle est effectuée (branchement d'une électrode ou d'une pince sur l'animal) la tension efficace de la stimulation ne doit pas excéder 25 volts. Cette valeur tient compte des courbes de sécurité publiées dans la norme NF C 15 p. 100. Cette valeur de 25 V efficaces est la tension maximale que peut supporter un être humain dans un local très humide.

Cependant, cette valeur est donnée soit en courant continu soit en courant alternatif à une fréquence de 50 Hz.

Il subsiste encore un doute quant au comportement humain supportant une série d'impulsions; les moyens de prévention sont encore à l'étude.

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Les conséquences socio économiques de la stimulation électrique et du désossage à chaud

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Parler des conséquences socio-économiques de la stimulation électrique et du désossage à chaud est un exercice difficile parce que ces deux nouvelles techniques surtout le désossage à chaud — en sont encore, en France, à un stade presque expérimental. En conséquence, leurs vastes conséquences socio-économiques ne peuvent être saisies qu'imparfaitement et plus pressenties et estimées que constatées et mesurées.

Dans un souci de simplification je ne distinguerais pas systématiquement dans la suite de cet exposé la stimulation électrique du désossage à chaud. En effet, dans le futur, il semble probable que ces deux techniques seront étroitement liées

Nous examinerons donc successivement :

1) Les avantages socio-économiques présentés par ces deux techniques.
2) Les contraintes que leur mise en œuvre implique.
3) Les principales perspectives qu'elles ouvrent.

1. — Les avantages présentés par ces deux techniques

Ils sont extrêmement nombreux. On peut citer :

1. — *Économie au niveau de l'investissement dans les nouvelles unités*

Cette économie provient principalement d'une sensible réduction du *volume des chambres froides* dans un système stimulation électrique + desossage à chaud, par rapport à la chaîne de traitement traditionnel des viandes :

— les surfaces sont identiques pour la contention, l'abattage des animaux et la préparation des carcasses;

— les salles de désossage à chaud manuel sont au moins aussi vastes que pour le désossage à froid;

— par contre le gain au niveau des chambres froides est important pour plusieurs raisons :

  — pas de réfrigération et ressuage des carcasses avant désossage et découpe;
  — stockage au froid de morceaux ou muscles sous vide — ce qui entraîne une diminution de l'espace occupé de 70 p. 100 par rapport aux carcasses.

G. HARRINGTON (Meat and Livestock Commission) a proposé une estimation de l'économie attendue au niveau de l'investissement pour une unité traitant 50 000 bovins/an.

Si le coût de l'investissement global est de 100 dans un système stimulation électrique + désossage à chaud, le coût est de 110 dans le schéma de désossage à froid. L'économie estimée au niveau de l'équipement frigorifique étant de plus de 30 p. 100.

Cette économie au niveau de l'investissement aura bien entendu des incidences positives sur les charges financières supportées par l'entreprise.

Deux remarques sur les coûts d'investissement

— si la stimulation électrique est seule pratiquée sans désossage à chaud, le fait de diminuer de manière sensible la durée de maturation des viandes permet de traiter, avec la même capacité frigorifique, un tonnage plus important de viande, et donc d'augmenter très nettement la capacité des unités d'abattage sans investissement supplémentaire;

— l'implantation de ces deux techniques dans des unités anciennes peut être à l'origine de dépenses relativement élevées dans la mesure où d'importantes modifications doivent être apportées dans la chaîne de traitement des viandes. C'est pourquoi l'implantation de ces nouvelles techniques serait surtout conseillée dans des unités nouvelles spécialement conçues à cet effet.

2. — *Économie d'énergie*

Dans le contexte que nous connaissons où l'énergie devient plus onéreuse, et sens doute à terme, plus rare, cet aspect doit retenir toute notre attention.

Les origines des économies de dépenses énergétiques attendues apparaissent avec évidence :

— absence de réfrigération des carcasses après abattage;

— forte diminution des volumes réfrigérés et stockés; absence d'os, de graisses et déchets;

— racourcissement des durées de stockage en raison d'un processus de maturation amélioré.

Les estimations de l'économie réalisée varient selon les experts mais sont toujours importantes : de l'ordre de 30 à 50 p. 100.

3. — *Amélioration du rendement pondéral des carcasses*

Le fait de désosser à chaud et de conditionner la viande immédiatement, réduit notablement les pertes par ressuage observées sur les carcasses traitées de manière traditionnelle. Selon les experts un gain de 1 à 2 p. 100 peut être envisagé.

Il semblerait en outre que ce gain pondéral ne se traduit pas par des pertes supplémentaires lors de la cuisson des viandes.

4. — *Amélioration de la qualité des viandes*

Cette question est encore controversée. Néanmoins de nombreuses observations ont mis en évidence l'effet positif de la *stimulation électrique* sur la tendreté des viandes (Suède — U.S.A. ...) et la diminution ou la disparition du phénomène de « cold shortening ».

L'O.N.I.B.E.V. finance actuellement dans un grand abattoir de l'ouest de la France des recherches qui ont pour but de préciser, en vue d'applications pratiques, les effets de la stimulation électrique sur la tendreté et la durée de maturation optimale des viandes. Cette expérience est en cours et a pour le moment mis en œuvre des stimulations électriques en basse tension. Les dégustations effectuées ont permis de constater que, malgré une diminution de 8 jours du temps de maturation la tendreté des viandes traitées était satisfaisante.

Cet aspect « tendreté » des viandes paraît très important car il correspond à une demande très générale des consommateurs. Ainsi la maîtrise de la stimulation électrique devrait permettre de faciliter les relations commerciales entre les transformateurs et les détaillants en supprimant les incertitudes sur la tendreté des viandes et sécuriser le consommateur dans ses achats.

On peut à ce sujet citer l'exemple de la Suède où une grande majorité de carcasses est maintenant stimulée et où des campagnes de publicité garantissent aux ménagères des viandes tendres et un remboursement en cas d'insatisfaction. Ces remboursements sont très rares.

Dans un système stimulation électrique + désossage à chaud, les améliorations de tendreté font également l'objet de controverses (cf. travaux de l'I.T.E.B.) En ce domaine, la cinétique du refroidissement a certainement un rôle déterminant.

Il paraît par contre acquis que la stimulation électrique a des effets très nets sur la présentation des viandes : couleur, persillé, graisse plus blanche et plus ferme, viandes moins exsudatives.

Dans le domaine qualitatif, il faut aussi souligner que le *désossage à chaud* peut apporter des améliorations sur le plan de l'hygiène des viandes. En effet, le désossage, le conditionnement et la réfrigération rapide des viandes aussitôt après l'abattage, en raccourcissant les délais de traitement et en supprimant beaucoup de manipulations, semblent améliorer la qualité bactériologique des viandes. Mais là aussi, des expérimentations sont nécessaires.

5. — *Meilleures possibilités de valorisation des carcasses*

Cette améliorations est due au fait que l'introduction des deux nouvelles techniques permet, dans une certaine mesure, un glissement qualitatif des découpes vers le haut de gamme et facilite le traitement industriel des produits de moindre valeur avec notamment incorporation de protéines récupérées (sang, abats).

Cet important aspect a été chiffré par HARRINGTON qui a retenu pour son évaluation un bovin de 455 kg /vif et 240 kg carcasse :

— dans le cas d'un désossage et conditionnement à froid 169 kg de découpes sont obtenus avec une valeur moyenne de 170 pence /kg;

— dans le cas d'un désossage et conditionnement à chaud et d'un traitement à chaud des sous-produits avec incorporation de produits du 5e quartier on obtient 195 kg de découpes, avec une valeur moyenne de 180 pence /kg.

Par rapport au traitement traditionnel le gain en poids est de 15 p. 100 et l'augmentation de la valeur des produits vendus est de 22 p. 100.

6. — *Économie de main-d'œuvre — pénibilité du travail*

Si on suppose que les problèmes liés à la nouvelle organisation du travail et aux nouvelles méthodes de désossage sont résolus, il est généralement admis que le procédé de désossage à chaud entraîne des économies de main-d'œuvre. En effet cette technique supprime beaucoup de manipulations de carcasses et de travaux qui interviennent sur la chaîne de traitement traditionnel.

Pour une unité traitant 50 000 bovins par an, HARRINGTON estime l'économie de main-d'œuvre à environ 8 p. 100 étant entendu qu'aucune économie ne peut être faite sur le personnel d'encadrement et sur le personnel chargé de l'abattage et de la préparation des carcasses.

En ce qui concerne la main-d'œuvre, il faut évoquer un aspect social important qui concerne la pénibilité du travail. Le désossage à chaud supprime en effet les opérations de portage à dos d'homme. En outre, les opérations de désossage n'ont plus lieu dans des locaux réfrigérés qui rendent le travail pénible. Le désossage à chaud pourrait donc permettre de proposer aux ouvriers de meilleures conditions de travail et par conséquent d'améliorer l'image de marque de la profession et d'élargir le recrutement en augmentant par exemple la représentation féminine dans les ateliers.

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Cette liste, déjà assez longue, des avantages de la stimulation électrique et du désossage à chaud, n'est sans doute pas exhaustive, ni suffisamment quantifiée, mais elle permet déjà de souligner tout l'intérêt présenté par ces nouvelles techniques qui, d'une manière très synthétique, devraient entraîner une diminution des coûts de production des entreprises liée à une amélioration de la qualité des produits.

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2. — Les contraintes socio-économiques imposées aux entreprises et aux hommes.

La stimulation électrique et le désossage à chaud ne peuvent s'implanter sans modification profonde des systèmes de production.

1) Il est nécessaire de mettre en œuvre un *nouvel agencement de la filière,* sujet qui a été développé par M. CERLES et sur lequel je n'insisterai donc pas. Je soulignerai toutefois que le désossage à chaud ne peut être mis en œuvre que dans des unités importantes puisque deux équipes doivent travailler simultanément.

2) *La stimulation électrique et le désossage à chaud nécessitent une bonne maîtrise technique* des nouveaux processus de transformation et notamment :

— les procédures de stimulation;
— l'évolution du pH des viandes et de leur température;
— le désossage et la préparation des muscles;
— l'état sanitaire des viandes;
— les durées de maturation;
— les problèmes posés par l'utilisation de ces viandes aux stades du détail et de la consommation.

Ces procédés nouveaux ne pourront être pleinement valorisés que si tous ces paramètres sont bien maîtrisés par les entreprises. Ceci implique également la mise en place d'un outil comptable en temps réel compte tenu de la grande dispersion des produits (qualité, poids, prix).

3. — *Au niveau de la main-d'œuvre*

Des difficultés pourraient intervenir dans la mesure où le désossage à chaud demande un savoir-faire très différent de celui qui est utilisé dans le désossage traditionnel. Une importante action de formation de la main-d'œuvre s'avère donc indispensable.

Parmi les contraintes je signale en outre pour mémoire l'importante question de la sécurité du personnel.

4. — *Modification de la présentation des viandes traitées*

Ces modifications porteront notamment sur la forme des morceaux et sur la couleur des viandes, éléments qui présentent une grande importance pour l'industrie des viandes puisqu'ils constituent des critères de sélection pour la vente au détail.

Une ample action auprès des utilisateurs de viandes stimulées électriquement et désossées à chaud sera donc nécessaire afin de les informer des particularités présentées par ces viandes et des avantages qu'elles présentent.

Des évènements récents, qui ont été très largement développés par les média, ont montré la nouvelle importance prise en France par les consommateurs et par conséquent la nécessité de les informer clairement et objectivement des nouvelles techniques introduites dans la filière.

5. — Enfin je crois qu'au niveau des contraintes, il est indispensable d'évoquer le *problème du financement* nécessaire au développement de la filière.

On sait qu'actuellement en France, les capacités d'investissement des industries de la viande sont très limitées (marge brut d'autofinancement proche de 1 p. 100 du chiffre d'affaires en année moyenne).

Cette fragilité financière est de plus peu incitative pour les investissements extérieurs au secteur.

L'introduction de ces deux nouvelles techniques dans l'industrie risque donc, dans une première étape, de poser des problèmes de financement des investissements.

Ne serait-ce que sur le plan financier elle s'avère pourtant à terme indispensable, dans la mesure où elle contribuera à restaurer les marges et donc à permettre un développement futur.

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* *

En conclusion on peut donc dire que l'implantation de ces nouvelles techniques implique trois principaux type d'action :

— une action de formation des hommes à tous les niveaux : gestion, encadrement, main-d'œuvre;

— une action d'information des utilisateurs en aval de l'atelier de découpe, y compris une information des consommateurs;

— une analyse des conditions de financement afin de permettre aux entreprises de faire face aux investissements nécessaires : investissements nécessités par le remodelage des unités actuelles ou la création de nouvelles unités adaptées aux deux nouvelles techniques à mettre en œuvre.

3. — Les perspectives ouvertes par la stimulation électrique et le désossage à chaud

1) La mise en œuvre de ces nouvelles techniques ouvre de vastes perspectives. Depuis une dizaine d'années en France, les secteurs de la transformation et de la distribution en gros des viandes sont en forte mutation passant d'un stade presque artisanal à un stade préindustriel. La stimulation électrique et le désossage à chaud devraient permettre d'obtenir un changement de rythme dans la modernisation et l'industrialisation de la filière en France, qui pourra ainsi montrer sa capacité à répondre aux besoins de nouveaux marchés, à créer de nouveaux produits et ceci, en tenant compte des nouvelles données de la production.

Quelques chiffres pourront nous permettre de mesurer les évolutions passées et de préssentir celles qui sont à venir :

Nombre d'abattoirs traitant des bovins (hors tueries particulières).

1970	1980 abattoirs
1979	690 abattoirs dont 26 de + de 10 000 T /an traitant 27 p. 100 de l'ensemble des abattages.

Évolution du nombre d'atelier de transformation des viandes

	1970	1979
Ateliers < 1 000 t	160	498
Tonnage traité	47 000 t	140 000 t
Ateliers > 1 000 t	36	128
Tonnage traité	78 000 t	405 000 t
Total ateliers	196	616
Tonnage traité	125 000 t	545 000 t soit 35 p. 100 environ de la production française de viande bovine

Dans les années à venir, l'implantation très probable de la stimulation électrique et à plus longue échéance du désossage à chaud accompagnera et facilitera la poursuite du processus de développement des entreprises industrielles tout en regroupant dans les mêmes unités les fonctions d'abattage et de désossage. Le pourcentage des viandes transformées devrait rapidement dépasser les 50 p. 100 de la production nationale.

2) Modification de l'approvisionnement de la distribution finale.

En 1978, les formes modernes de distribution s'approvisionnaient pour :

60 p. 100 en carcasses;
30 p. 100 en muscles et pièces sous vide;
8 p. 100 en viandes piécées conditionnées;
2 p. 100 en viandes hachées réfrigérées ou surgelées.

La stimulation électrique et le désossage à chaud entraîneront un renforcement très net des approvisionnements des formes modernes de distribution en muscles et pièces conditionnés au détriment des carcasses.

Au niveau de la restauration commerciale et collective qui représente actuellement près de 20 p. 100 de la viande consommée en France, une évolution du même type devrait s'observer.

Enfin la stimulation électrique et le désossage à chaud pourraient également contribuer à infléchir les modalités d'approvisionnement et les méthodes de travail de la boucherie traditionnelle.

3) *Développement de nos échanges extérieurs — Reconquête du marché intérieur français*

Dans la communauté européenne, les viandes françaises doivent faire face à une concurrence très vive, aussi bien sur le marché national qu'à l'extérieur de nos frontières. Et on ne peut dire que depuis quelques années, cette confrontation ait tourné à notre avantage.

Cette situation peut être illustrée par quelques chiffres :

De 1970 à 1976, le commerce extérieur français en viandes bovines fraîches est resté pratiquement équilibré. Un déficit est apparu à partir de 1977 et il n'a fait que se creuser depuis pour atteindre 60 000 tonnes en 1979.

On constate parallèlement un déséquilibre croissant entre les échanges de quartiers avant et les échanges de quartiers arrière.

En 1974, le solde de nos échanges de quartiers avant était positif de 180 000 tonnes, en 1979, il n'a plus été positif que de 34 000 tonnes. Par contre, les échanges français de quartiers arrière n'étaient déficitaires que de 18 000 tonnes en 1974; ce déficit dépasse 140 000 tonnes en 1979.

Devant ce constat, on peut dire que tout ce qui va dans le sens d'une amélioration de la compétitivité des entreprises françaises et d'une industrialisation plus poussée des opérations de transformation, permettant de mieux valoriser les quartiers avant, est souhaitable.

C'est pourquoi, l'introduction de la stimulation électrique et du désossage à chaud doit être considérée comme une priorité dans le développement de nos entreprises. Ces deux techniques doivent permettre d'obtenir des viandes de qualité, de présentation homogène et à des prix plus compétitifs, susceptibles de mieux valoriser l'ensemble des carcasses, adaptées aux nouvelles demandes nationales et internationales et permettant aux entreprises de définir et de mettre en œuvre

des politiques commerciales cohérentes et ambitieuses, incluant par exemple des politiques de marques adaptées aux nouvelles gammes de produits.

4) *Possibilités d'introduire d'autres technologies nouvelles*

Elles doivent être évoquées car seules la mécanisation et l'automatisation des opérations de désossage et de découpe pourraient induire d'indispensables économies d'échelles.

En France, la recherche d'une mécanisation des opérations de désossage a fait l'objet d'un certain nombre d'idées, de brevets et même de prototypes : qu'il s'agisse de jets coupants, de bistouris électriques ou d'ultra-sons.

Mais nous sommes encore assez loin des applications industrielles.

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